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(54) Title: INHIBITORS AND TARGET MOLECULE CO-LOCALIZATION

(57) Abstract

The invention provides mechanisms for the co-localization in a living cell of a target molecule and of an inhibitor for the target molecule. The invention also provides novel chimeric $tRNA^{LYS}$-ribozyme molecules that compete effectiely with $tRNA^{LYS}$ for HIV-1 reverse transcriptase binding sites. The chimeric human $tRNA^{LYS}$-ribozymes inhibit reverse HIV transcription by delivering inhibitors such as ribozymes of HIV-1 reverse transcriptase directly to the virion particle and render it non-functional. The chimeric molecules of the invention thus serve as highly specific non-toxic therapeutic agents and vaccines for viral, including lentiviral, infections. These chimeric molecules also reveal a novel, site specific RNA cleaving activity of HIV-1.

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INHIBITORS AND TARGET MOLECULE CO-LOCALIZATION

This invention was made with government support under Grant No. AT 25959 awarded by the National Institutes of Health. The government has certain rights in the invention.

This application is a continuation-in-part of Serial No. 08/185,827 filed January 24, 1994.

FIELD OF INVENTION

This invention relates to mechanisms for bringing two or more molecules together in a living cell. More particularly, the invention relates to mechanisms for bringing together with a cell a target molecule and an inhibitor therefore in a manner effective to increase the concentration of the inhibitor with respect to the target. For example, the invention relates to mechanisms for increasing the cellular concentration of a ribozyme with respect to a target mRNA molecule to be cleaved by the ribozyme.

One embodiment of this invention relates to chimeric $tRNA^{LYS}$ ribozyme molecules which compete effectively with $tRNA^{LYS}$ for binding to HIV-1 reverse transcriptase. These chimeric molecules provide a co-localization mechanism for delivering inhibitors of HIV-1 and reverse transcriptase to the virion particle itself.

BACKGROUND OF THE INVENTION

RNA is unusual in its ability both to store information in its nucleotide sequence and to function as an enzymatic catalyst of specific reactions (1,2). This combination of attributes has created opportunities for engineering RNA enzymes (ribozymes) which can be used to cleave and functionally inactivate targeted RNAs. Some of the attributes of ribozymes which make them attractive candidates for therapeutic agents are their ability to site-specifically cleave targeted RNAs, cleave multiple substrates, and their ability to be engineered for improved cleavage specificity and enhanced catalytic turnover (3,4). There are five catalytic motifs which have been successfully modified and/or adapted for use in

ribozyme applications. These are the group I introns, RNAse P, the hammerhead and hairpin motifs, and the self-cleaving domain of the hepatitis delta virus (5,3,6,7). Each of these engineered ribozymes only requires a divalent metal cation for activity (usually MG^{++}) which participates in the chemistry of the cleavage reaction (8,9,10).

The therapeutic use of ribozymes is an attractive goal which merges the basic and applied sciences. Since all genes are expressed through RNA intermediates, the potential applications are primarily limited by knowledge of the disease or disease associated with a given RNA. In the case of viral infections, such as HIV, ribozymes can be tailor made to cleave viral transcripts, thereby leaving cellular transcripts untouched. Because of this, HIV is a prime target for ribozyme inactivation. This concept was successfully tested by intracellularly expressing a hammerhead ribozyme targeted to a gag cleavage site, which resulted in up to a 40 fold reduction in viral p24 antigen production in HeLa CD4+ cells challenged with HIV (11,12). As a retrovirus with an RNA genome, there are hundreds of potential ribozyme cleavage sites along the length of the viral genomic and subgenomic RNAs. Since the virus mutates rapidly, and can become resistant to most drugs developed to inhibit a single viral target (13), ribozymes have become an important alternative for anti-viral therapeutic agents since multiple ribozymes targeted to a number of different sites can be simultaneously delivered to cells for inhibition of HIV (14). There are two times in the viral life-cycle when ribozymes could be effective against HIV infection. The first immediately following infection prior to proviral DNA formation, when all or part of the viral genome is still in the form of RNA, and the second following the establishment of integrated provirus from which spliced and a full length viral

transcripts are produced (15,16). An important consideration is the observation that HIV can infect quiescent T-lymphocytes, wherein proviral DNA synthesis is initiated but is incompletely reverse transcribed (17). If a ribozyme is present in the infected cell cytoplasm, it theoretically could protect cells from permanent infection by cleaving the RNA at this early step, before the T-cell becomes activated. In support of this type of action by a ribozyme, Yamada, et al., (18) have demonstrated a 50-100 fold reduction in HIV proviral DNA formation in cells expressing a hairpin ribozyme targeted to a site in the 5' leader sequence.

A number of reports demonstrating varying levels of ribozyme mediated protection of cultured cells from HIV infection have been published (14,19,20,21,22,12,23,24). The most encouraging results of ribozyme mediated inhibition of HIV utilized a hairpin ribozyme targeted to a highly conserved GUC cleavage site in HIV (22,18,24). Expression of this ribozyme gave rise to long term resistance to infection, including resistance to a variety of HIV isolates. Studies recently completed demonstrate that hammerhead ribozymes targeted to conserved sites in the tat and a shared tat-rev exon, when expressed from a Moloney viral vector LTR can confer protection to cells in culture for at least 21 days (Gene 149:33-39 (1994), incorporated herein by reference). Despite these reported successes, the observation has been made that ribozyme mediated protection of cells can be overcome with increasing multiplicity of infection, and in some instances with prolonged culture items. In a patient setting, this is likely to be a serious problem since there is substantial evidence suggesting that the virus is highly concentrated in the lymphoid system, providing in essence, a high multiplicity of infection to CD4+ cells entering that environment (25,26,27). A somewhat different problem is that of the genetic

variability of HIV (15). Although it has not been formally demonstrated in experimental models, it is reasonable to assume that viral resistance to ribozymes can and will occur, especially in a patient setting, where the pool of viruses is bound to be genetically heterogeneous (28,13,29,30).

The first steps in solving this problem involve developing a detailed understanding of how ribozymes can be made to function more effectively in an intracellular environment. For most RNAs, very little is known about the mechanisms regulating the pathway of movement from transcription through translation, and in the case of HIV, from transcription through packaging. There is increasing evidence, although some of it still controversial, that nuclear transcripts are processed and migrate along specific tracks, which predicts non uniform distributions of specific nuclear transcripts (31). Following export from the nucleus, there is also increasing evidence that a variety of RNAs can be specifically localized within the cytoplasm as well (32). From the prospective of ribozyme therapeutic applications, capitalizing upon the localization properties of RNAs could facilitate intracellular functioning of ribozymes by allowing them to co-localize with their target RNAs. Sullenger and Cech (1993) (33) (incorporated herein by reference) have directly tested this idea by utilizing the dimerization and packaging signal of a Moloney murine leukemia virus genomic RNA to co-localize a hammerhead ribozyme with its target, the lac Z gene carried by another recombinant Moloney viral vector. They found that up to 90% inhibition of infective virus production could be achieved as a result of co-packaging the ribozyme and the lacZ target containing viral RNAs. Their data showed that inhibition of lacZ expression was only achieved when the ribozyme was co-packaged with the genomic target RNA. Thus, mRNAs

harboring the lacZ sequence, but lacking the packaging signal, were not destroyed by the ribozyme, clearly demonstrating the usefulness of a co-localization strategy for ribozymes.

A different co-packaging strategy which takes advantage of the fact that HIV utilizes $TRNA^{LYS3}$ as a site specific primer for reverse transcription is described in U.S. Serial No. 08/185,827. A ribozyme capable of pairing with and cleaving HIV at a site just upstream of the primer binding site to the 3' end of $tRNA^{LYS3}$. The strategy in that application is that the chimeric molecules could be bound by HIV reverse transcriptase, and captured during viral assembly.

During the series of events that RNAs undergo from their birth to their death, they are constantly associated with proteins (34). It is a virtual certainty that ribozymes will encounter proteins in an intracellular environment which will have an effect, either positive or negative, on their activity. Tsuchihashi, et al. (1993) (35), Herschlag, et al., (1994) (36) and Bertrand and Rossi (1994) (37) have observed RNA binding proteins such as HIV-1 encoded NCp7 and cellular hnRNP A1 can facilitate ribozyme catalytic turnover in vitro.

The ribozyme-target co-localization strategy described in Serial No. 08/185,827 involves utilizing the $tRNA^{LYS3}$ primer for reverse transcriptase (RT) as a vehicle for co-localizing a ribozyme with HIV genomic RNA, and potentially into the virion itself. The strategy is based upon the well established interactions of HIV RT with cellular $tRNA^{LYS3}$, which is the primer tRNA used by all the mammalian lentiviruses. This tRNA is selectively bound by RT, and in the presence of the nucleocapsid protein NCp15 (or NCp7), unwinds the aminoacyl stem of the tRNA, allowing it to base pair with the viral PBS (38). The premise of Serial No. 08/185,827 is that a

ribozyme appended to the 3′ terminus of $tRNA^{LYS3}$ could be captured by RT, co-packaged with the virus, and the ribozyme would be aligned to cleave the viral genomic RNA and destroy its infectivity. Available data supports the hypothesis. These data are summarized as follows: (1) The tRNA-ribozyme binds selectively to HIV RT with a binding affinity virtually identical to a synthetic $tRNA^{LYS3}$. (2) The tRNA-ribozyme is expressed as a Pol III transcript when transfected into 293 cells, and the ribozyme moiety is not processed from the transcript, although the 5′ precursor segment of the tRNA-ribozyme is processed normally. By including the CCA in the transcripts, which is normally added post-transcriptionally to the tRNA, these molecules are not subject to the normal 3′ processing events. (3) The tRNA-ribozyme is exported to the cytoplasm, making it available for binding with RT. (4) When the tRNA ribozyme is transiently transfected into 293 cells, there are equivalent levels of tRNA-ribozyme transcript to endogenous $tRNA^{LYS3}$. (5) Co-transfection of the tRNA-ribozyme gene with pNL4-3 DNA into 293 cells resulted in a 4 to 12 fold reduction in infectious virus production relative to control constructs. See Figure 5.

It has been demonstrated that the entire $tRNA^{LYS}$ molecule as well as various segments of the tRNA per se are capable specifically of interacting with HIV-1 transcriptase. See Barat, et al. EMBO Journal 8:3279-3285 (1989); Khan, et al. J. Bio. Chem 267:6689-6695 (1992); Weiss, et al., Gene 111:183-197 (1992). Ben-Artzi, Proc. Natl. Acad. Sci. USA 89:927-931 (1992) reports an RNAse cleavage activity associated with HIV-1 reverse transcriptase. This activity is shown to cleave only HIV-1 RNA, not the primer.

Prior to this invention there has been no report of chimeric $tRNA^{LYS}$-ribozyme molecules.

DEFINITION

Co-localization: As used in this application, the term co-localization means the positioning of two or more molecules within a living cell, one of which is a target and the other an inhibitor of the target that the concentration of the inhibitor with respect to the target is increased within the cell and function or expression of the target is constrained or inhibited.

Co-localization may be accomplished by covalent linkage (cis-ribozyme) or via co-targeting the viral capsid. A specific embodiment of co-localization pursuant to this invention entails the positioning within a living mammalian cell of a ribozyme adjacent a virion particle to cleave virion RNA.

SUMMARY OF THE INVENTION

This invention provides co-localization mechanisms and living cells in which an inhibitor and a target are co-localized by such mechanisms. An important object of the invention is to provide novel intracellular immunogens for vaccines against viral infections.

One preferred embodiment of this invention provides novel chimeric $tRNA^{LYS}$-ribozyme molecules that compete effectively with $tRNA^{LYS}$ for HIV-1 reverse transcriptase binding sites. The chimeric human $tRNA^{LYS}$-ribozymes inhibit reverse HIV transcription by delivering inhibitors such as ribozymes of HIV-1 reverse transcriptase directly to the virion particle and render it non-functional. The chimeric molecules of this invention thus serve as highly specific non-toxic therapeutic agents.

These chimeric molecules also reveal a novel, site specific RNA cleaving activity of HIV-1.

DESCRIPTION OF THE FIGURES

Figure 1 (SEQ ID NO. 1 and SEQ ID NO. 2) shows the structure of one chimeric ribozyme. This $tRNA^{LYS}$-ribozyme construct has been cloned into a Blue Script

transcription vector using SacII and XhoI restriction sites. Following linearization at the SacII site the chimeric RNA can be transcribed in vitro using bacteriphase T-7 RNA polymerase. There is also a Mae I restriction site in between the tRNA and ribozyme moieties, allowing the tRNA to be transcribed independently of the ribozyme.

Figure 2. This gel shift experiment shows binding of the chimeric $tRNA^{LYS}$-ribozyme to HIV-1 reverse transcriptase. The eight lanes of the gel from left to right are:

1. Molecular weight marker.

2. $tRNA^{LYS}$ in vitro transcript which has extra bases at both the 5′ and 3′ ends. The extra 5′ bases are from the Blue Script poly linker between the T-7 promoter and the XhoI site. There are six extra nucleotides at the 3′ derived from the nucleotides after the CCA of the tRNA to the Mae I site which separates the tRNA from the ribozyme.

3. $tRNA^{LYS}$-ribozyme in vitro transcript which has the same extra 5′ bases as $tRNA^{LYS}$, but terminates at SacII site at the end of the ribozyme moiety.

4. $tRNA^{LYS}$-transcript incubated with HIV-1 reverse transcriptase.

5. $tRNA^{LYS}$-ribozyme transcript incubated with HIV-1 reverse transcriptase.

6. $tRNA^{LYS}$-transcript incubated with AMV reverse transcriptase.

7. $tRNA^{LYS}$-ribozyme incubated with AMV reverse transcriptase.

8. $tRNA^{LYS}$ with competing, non-radioactively labelled tRNALYS-ribozyme incubated with HIV-1 reverse transcriptase.

This Figure 2 shows that the chimeric $tRNA^{LYS}$-ribozyme specifically binds to HIV-1 reverse transcriptase by a shift in radioactivity when HIV-1

reverse transcriptase is present. Cold $tRNA^{LYS}$-ribozyme competes with $tRNA^{LYS}$ for binding to HIV-1 reverse transcriptase as indicated by the reduced radioactive shift in lane 8.

Figure 3. This experiment demonstrates cleavage of a 162 nucleotide, radioactively labelled HIV-1 RNA containing the primer binding site plus sequences upstream of this and including the AUC cleavage signal for the ribozyme. The cleavage products are 101 and 61 bases. The extent of cleavage increases with increasing temperature.

Figure 4. Demonstration of the novel RNAse activity of HIV-1 reverse transcriptase when $tRNA^{LYS}$-ribozyme and HIV-1 primer binding site transcripts are incubated together in the presence of HIV-1 reverse transcriptase. The $tRNA^{LYS}$-ribozyme is radioactively labelled, and the HIV-1 RNA is non-radioactive. The cleavage products result in the tRNA moiety being separated from the ribozyme moiety. This result also demonstrates that the chimeric $tRNA^{LYS}$-ribozyme cannot serve as a primer for HIV-1 reverse transcriptase.

The lanes are, left to right: $tRNA^{LYS}$-ribozyme alone, $tRNA^{LYS}$-ribozyme plus HIV-1 reverse transcriptase, no deoxyribonucleoside triphosphates; $tRNA^{LYS}$-ribozyme plus HIV-1 reverse transcriptase plus deoxyribonucleoside triphosphates; last two lanes same as lane 3 except lane 4 has AMV reverse transcriptase and lane 5 has MLV reverse transcriptase. The black dots mark the HIV-1 reverse transcriptase cleavage products. Unlabelled HIV-1 primer binding site containing 162 nucleotide transcript was present in each lane. None of the reverse transcriptases can utilize the $tRNA^{LYS}$-ribozyme as a primer since it has 12 nucleotides at the 3' end which cannot base pair with the HIV-1 primer binding site RNA.

Figure 5. Illustrates A: RT binding to $tRNA^{LYS3}$-ribozyme. B: Primer extension analyses demonstrating

nuclear localization of chimeric transcript. The primer for the tRNA-ribozyme is in the ribozyme moiety, and the primer for $tRNA^{LYS3}$ is at the 3' end of the tRNA.
C: Results of infectious virus assays carried out with supernatents from 293 cells transfected with tRNA-ribozyme or control construct (ribozyme minus tRNA in same vector) and co-transfected with pNL4-3. Three independent experiments are presented.

Figure 6 illustrates the $tRNA^{Lys3}$-ribozyme which is the starting molecule. The asterisks indicate sites which UV crosslink to HIV RT or are protected from RNAse digestion in the presence of RT. A deliberately created mismatch in the ribozyme pairing arm is indicated with a boxed in nucleotide pair. This was done to eliminate a stretch of 4T's in the ribozyme gene which could serve as a Pol III termination site. The authentic termination site (5 U's or T's in DAN) is underlined. The T loop-stem and aminoacyl acceptor stem which pair with the HIV primer binding site are overlain with a heavy line.

Figure 7 is a schematic representation of nef and 3'UTR region to be included in ribozyme and GH reporter systems. The delineating sequences are the extremities of the DNA amplified by PCR. These sequences are from the pNL4-3 proviral clone and encompass the region of nucleotides 9389 through 9704.

Figure 8 represents a construct containing anti-HIV-1 ribozyme expressed in context of dimerization domain and RRE to facilitate co-localization with HIV full-length genomic RNAs.

GENERAL DESCRIPTION OF THE INVENTION

The invention provides various co-localization mechanisms. These mechanisms include, among others, (i) utilization of specific RNA trafficking pathways to both the target and the inhibitor, (ii) utilization of protein interaction with inhibitor and target molecules, e.g., HIV-1 RT (see Sullenger and Cech (33)), (iii) use

of cellular proteins which subcellularly compartmentalize the inhibitor to the target or a specific target site; (iv) use of cis-acting sequence substituents on ribozyme transcripts to direct the ribozyme to a specific subcellular trafficking pattern or site; (v) ribozymes which include any molecule or moiety that specifies a distinct intracellular trafficking pattern and target localization site.

1. Co-Localization of Ribozymes with HIV-1 or Cellular RNA Targets.

a. $tRNA^{LYS3}$-ribozyme chimeric molecules

One of the most important problems facing the routine use of ribozymes as therapeutic agents is that of maximizing effective interactions of ribozymes and target RNAs. It has been convincingly demonstrated by Sullenger and Cech (33) that co-localization of a ribozyme and target RNA through a retroviral packaging signal can dramatically enhance the effectiveness of the ribozyme pairing with, and cleaving its substrate. As noted, Serial No. 08/185,827 describes somewhat different co-localization strategy with the $tRNA^{LYS3}$-ribozyme chimeras (see Progress Report section), which are bound by HIV reverse transcriptase allowing alignment of the ribozyme during packaging of the virus. This approach has been successful and has led to a reduction of infective viral titer as a consequence of co-expressing chimeric tRNA-ribozymes with HIV proviral DNA. In order to make this a more generally useful strategy, it is useful to develop chimeric molecules which effectively compete against cellular tRNAs for binding to RT, yet do not create a general toxicity problem. One of the goals of this invention is to develop genetic variants of $tRNA^{LYS3}$ which maintain the sequence and structural features required for interaction with a ribozyme for cleavage, yet are dissimilar enough from cellular $tRNA^{LYS3}$ so as not to interfere with normal cellular metabolism. The use of

these variants will also be coupled with enhanced intracellular expression systems. The identification of molecules which can still interact with the primer binding site of HIV (which means leaving at least the 3' segment of the amino-acyl acceptor stem intact), thereby allowing alignment of a ribozyme (appended to the 3' end) with a cleavage site adjacent to the viral primer binding site is contemplated.

Since high levels of expression of the $tRNA^{LYS3}$-ribozyme chimeric gene during transient transfection were observed, it is reasonable that inserting multiple, tandem copies of the tRNA ribozyme chimeric genes in a vector such as adeno associated virus (AAV) can also lead to high level expression.

A potential strategy for increasing the intracellular levels of the chimeric ribozyme transcript is to express them from heterologous promoters. For those variants which lack the A or B boxes, this will be a necessity. For variants which have maintained these elements, site directed changes which eliminate the promoter function will allow testing of these constructs using heterologous promoters. Several candidate promoters have been developed for ribozyme expression. The human U6 snRNA gene has a Pol III promoter element which is 5' of the coding sequence (Parry, et al. (39)). Transcription terminates after a string of 5 Uracil residues, resulting in a RNA with well defined ends. It has been demonstrated that this promoter can be used to transcript ribozyme containing RNAs which localize to the cytoplasm. A potential advantage of this promoter is that transcription can be engineered to initiate at the +1 sequence of the tRNA molecule, thus eliminating any need for processing a 5' leader, and allowing the synthesis of a very defined transcript.

b. The 3' untranslated region (UTR) as an RNA trafficking signal-model for ribozyme-target co-localization.

The factors which dictate the trafficking and intracellular localization of RNAs are poorly understood. There are some reports which suggest that RNAs may "track" along specific paths following transcription and transport to the cytoplasm (reviewed in 31). There are numerous examples of messenger RNAs which localize to specific regions of the cytoplasm as well. The most well studied localized RNAs are the oocyte and early embryo mRNAs of Drosophila and Xenopus (32). Other mRNAs such as actin have been shown to localize to cytoskeletal components (40, 41, 42). The signal for localization for many of the mRNAs which have been studied resides in the 3' untranslated region (32,42). Given that knowledge is limited as to how and why some mRNAs are localized to specific sub regions of the cytoplasm, for the majority of targets it is difficult to design ribozymes which will be at the right place in the cell to maximize interactions with a given target RNA. Kislauskis, et al. (42) have demonstrated that the mRNAs encoding two actin isoforms, β-cytoplasmic and α-cardiac, can occupy different cytoplasmic compartments within the same cytoplasm of chicken fibroblasts. Moreover, the sequences in the respective actin 3' UTRs were sufficient to localize a lac Z mRNA to the same cytoplasmic compartments. Actin isoforms contain very few differences in amino acid coding sequences, but the 3' UTR's are isoform specific, and evolutionarily conserved within a given isoform family, suggesting an important functional role (43). In order to demonstrate the utility of co-localizing a ribozyme transcript with a given mRNA, the β-actin and α-actin UTR's may be used to test their potential for co-localizing ribozyme and target mRNA's intracellularly. A similar approach

involves using the HIV-1 3′ UTR, which is present in all HIV transcripts.

The basic strategy is to incorporate the 3′ UTR of interest onto a reporter construct as well as to incorporate the same UTR onto a ribozyme transcript. The 293 and HeLa cell lines were used for the studies. The reporter construct to be used is depicted below and contains the human growth hormone (GH) gene driven by the SIV-1 LTR promoter. This system produces a readily quantifiable (using a radioimmunoassay) secreted protein. The linear range of response of GH expression to plasmid concentration in the 293 cell line was established. The expression of this construct is not dependent upon TAT expression, although a 10 fold stimulation of expression in the presence of SIV TAT was observed. If the results look promising in the 293 cell line, confirmation testing in HeLa cells will be carried out. The 3′ UTRs will be appended to both the growth hormone and ribozyme expression cassettes. To do this, the human β-actin or α-actin 3′ UTRs will be isolated from human genomic DNA or mRNAs utilizing PCR.

The primers for isolating the two human actin 3′ UTRs are:

beta actin oligo 5′
5′AGATCTTCTAGACCCGGGTAGGCGGACTATGACTTAGTTGC3′
(SEQ ID NO. 3)

beta actin oligo 3′
5′GAATTCGCTAGCTACGTACCCACCCTCTGCTGCCCCCAAC3′
(SEQ ID NO. 4)

alpha actin oligo 5′
5′AGATCTTCTAGACCCGGGCTAAGATGCCTTCTCTCTCCATC3′
(SEQ ID NO. 5)

alpha actin oligo 3′
5′GAATTCGTCAGCTACGTAACAATGCTCAGGGTGTCAAAGCA3′
(SEQ ID NO. 6)

The ribozyme will be expressed utilizing the RSV promoter with the appropriate actin UTR appended to the 3′ end. Utilizing transient transfection of the reporter constructs into pools of stably transfected ribozyme containing cells, the effect of the ribozyme mediated inhibition of the reporter construct was monitored. Ribozyme constructs may be made in the adeno associated virus vector backbone. The constructs will be encapsidated in collaboration with Saswati Chatterjee's laboratory, and transduced into three A293 or HeLa cell lines. Stable lines will be selected from G418, and levels of ribozyme expression will be monitored via primer extension and northern gel analyses. For each ribozyme, a non-cleaving mutant control will be used. The controls for 3′ UTR effects will utilize comparison of the efficiency of reporter gene inhibition as a function of having the β- versus α-actin 3′ UTRs, which localize to different intracellular compartments, appended to the reporter and ribozyme transcripts. Several ribozyme targets in the SIV leader region have been established which will be tested in conjunction with the UTRs. These ribozymes have been tested for substrate interaction using an in vitro gel shift assay, and identified by this process sites in the SIV LTR which are most accessible to binding. In each case where binding was shown to be efficient, good cleavage activity by the ribozyme was observed.

At this time, aside from the well known actions of Rev on RRE containing transcripts, there is very little known about the role, if any, of the HIV UTR on intracellular partitioning of messenger RNAs. The nucleotide sequence of the region is uninformative, but the functions of the LTR, such as transcription termination and polyadenylation signaling, must be conserved. Since the 5′ and 3′ LTRs of retroviruses are identical, but have functionally different roles

(transcription initiation for the 5′ LTR and termination and 3′ processing for the 3′ LTR), it is reasonable to ask whether placing a segment of the LTR at the 3′ end of a heterologous transcript will result in its functioning as a transcriptional termination, polyadenylation signal. An intact HIV-1 LTR has been appended to the 3′ end of an insulin reporter gene and more than 98% of the transcripts were correctly processed and polyadenylated at the authentic poly A site (44, 45). It is therefore reasonable to test this region for its potential use as an mRNA localization signal. The following experiments are illustrative.

The first set of sequences appended to the GH reporter construct included the last 20 bases of the pNL4-3 proviral<u>nef</u> coding sequence and extended to the 3′ terminus of the LTR. Much of this region is included in all of the viral messenger and full length genomic transcripts. This sequence contains the poly A additional signal and putative transcriptional termination region (45), but most importantly lacks cis-acting regulatory signals such as the RRE, INS and CRS. This region was isolated using PCR primers and appended to both the GH reporter gene construct and the ribozyme transcriptional units as described above.

The control constructs included the AAV poly A and termination signals, which were appended to the ribozyme and GH reporter constructs as well as mutant, non-cleaving ribozymes. Again, efficacy was measured by inhibition of growth hormone secretion in transient transfection assays of the GH construct into stable cell lines expressing the ribozyme constructs as described above.

c. Co-localization of anti-HIV-1 ribozymes with full length viral transcripts via the dimerization domain and the viral RRE.

The third strategy for co-localizing ribozyme and target RNAs will capitalize upon the unique RNA-RNA interaction of the dimerization domain of HIV (which is facilitated by the NCp7 nucleocapsid protein) (46-49) in combination with the RRE (to force cytoplasmic translocation of the ribozyme containing transcripts). The rationale for these studies is that ribozyme containing RNAs which harbor the signals required for packaging can be co-localized with unspliced viral mRNAs and genomic RNAs via interactions of the dimerization domains. The most probable targets for ribozyme interactions will be full-length viral RNAs, destined for encapsidation or translation into viral structural proteins. These experiments are based upon the success of a somewhat similar strategy employed by Sullenger and Cech (33). See Figure 8.

Genetic fusions consisting of the entire mature coding sequence or 18 bases of the 3′ end of human $tRNA^{LYS}$ were fused to hammerhead ribozyme containing RNAs with base pairing capabilities to the HIV-1 sequences immediately 5′ or upstream of the primer binding site. The 3′ terminal 18 nucleotides of the $tRNA^{LYS}$ are complementary to the primer binding site.

These chimeric molecules have been tested in cell free assays for their ability to bind to HIV-1 reverse transcriptase and their inhibitory activity on HIV-1 reverse transcriptase polymerization activity. The ribozyme moiety targets the cleavage of HIV-1 viral RNA at a known hammerhead cleavage site immediately upstream of the primer binding site for initiation of reverse transcription in the HIV-1 viral RNA. The site chosen for initial study, and reported here is an AUC in which cleavage is immediately after the C. This site is

absolutely conserved in all HIV-1 isolates sequenced to date. The chimeric RNAs, which are specifically bound by HIV-1 reverse transcriptase, should be carried into newly formed HIV-1 virions during viral assembly. The chimeric primers effectively block HIV-1 revere transcription, making them a novel, highly target specific, and unique anti-HIV-1 therapeutic agent. In addition, the $tRNA^{LYS}$ portion contains within its mature coding sequence the elements required for transcription by human RNA polymerase III, thereby making it feasible to insert the gene, rather than the RNA, into human cells.

Studies of the binding of the chimeric molecules to HIV-1 reverse transcriptase revealed that the complex of chimeric $tRNA^{LYS}$-ribozyme, or 18 3′ nucleotides of $tRNA^{LYS}$-ribozyme, or $tRNA^{LYS}$ with an extra 6 nucleotides appended to the 3′ end, when base paired to the primer binding site signal of HIV-1 RNA, serves as a substrate for a novel ribonuclease activity associated with HIV-1 reverse transcriptase. This activity results in cleavage of the primer at a site very close to the 3′ end of the $tRNA^{LYS}$ molecule, CCA-3′. This activity is of unknown function in the viral replication cycle, but may play an important role in the use of chimeric RNAs by freeing the ribozyme moiety from the tRNA moiety such that it can cleave one or both of the viral RNAs encapsidated in the HIV-1 virion.

GENERAL PURPOSE OR UTILITY OF THE INVENTION

Co-equalization mechanisms and the resulting living cells which include co-equalized inhibitors and targets are disclosed. HIV and other lentiviral RNAs co-equalized with a ribozyme provide intracellular and therapeutic agents and vaccines for mammalian lentiviral infections. Such therapeutic agents and vaccines are administered in known manner by viral mediated delivery, e.g., AAV or retroviral deliveries.

The idea of chimeric $tRNA^{LYS}$-ribozyme molecules which effectively compete with $tRNA^{LYS}$ for binding to HIV-1 reverse transcriptase is novel. It provides a possible mechanism for specifically delivering inhibitors of HIV-1 reverse transcriptase to the virion particle itself. Such inhibitory agents will render these viral particles non-functional, and thus serve as highly specific, non-toxic therapeutic agents.

It has been demonstrated that the entire $tRNA^{LYS}$ molecule, as well as various segments of the tRNA itself, are capable of specifically interacting with HIV-1 verse transcriptase. No one has shown that chimeric molecules such as the ones described could specifically bind to HIV-1 reverse transcriptase polymerase activity. There is one published report of an RNAse cleavage activity associated with HIV-1 reverse transcriptase. This activity was only shown to cleave HIV-1 RNA, not the primer. This activity cleaves twice in the primer binding site, and only substrates paired with $tRNA^{LYS}$.

The RNA attached at the 3' end of the $tRNA^{LYS}$ need not be a ribozyme, but any extra RNA which can base air with the HIV-1 target upstream of the primer binding site. If a ribozyme is joined to the tRNA, other cleavage sites such as CUC, or CUA which are on the HIV-1 sequence just to the 3' side (downstream) of the AUC site, can be targeted. It is not necessary to make an entire $tRNA^{LYS}$-ribozyme fusion because it is now known that the last 18 nucleotides of $tRNA^{LYS}$ fused to the ribozyme are also bound by HIV-1 reverse transcriptase. Genetic variants of $tRNA^{LYS}$ which compete better than $tRNA^{LYS}$ for binding to HIV-1 transcriptase are included in the invention.

The ribozyme fusions to $tRNA^{LYS}$ allow specific targeting of the ribozyme to HIV-1 virion. Since all retroviruses use cellular tRNAs for priming, this invention provides a general strategy for inhibiting

other retroviruses as well. Existing ribozyme technology makes use of specific base pairing between ribozyme and target, but this is accomplished by diffusion of the ribozyme until it finds a target RNA. This invention uses well known retroviral packaging pathways to specifically carry the ribozyme into the virion, and get it bound to the correct site on the viral RNA for cleavage.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Garry P. Larson

(ii) TITLE OF INVENTION: Inhibitors and Target Molecule Co-Localization

(iii) NUMBER OF SEQUENCES: 6

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(A) MEDIUM TYPE: Wang Double Density 5 1/4" diskette

(B) COMPUTER: Wang PC

(C) OPERATING SYSTEM: MS-DOS (R) Version 3.30

(D) SOFTWARE: Microsoft (R)

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(C) TELEX: None

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

UGGAAAAUCU CUAGCAGUGG CGCCCGAACA GGGAC 35

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 130

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

GGCGCCUUUU UACCGUUUAA AGCAGGAGUG CCUGAGUAGU CAGAUCGUCA 50
CCGCGGGCUU GUCCCUGAAC UUGGGACCUG GGAGUCUAAU UUUCAGACUA 100
CGAGAUGGCU GACUCGAUAG GCCCGAGCUC 130

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

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AGATCTTCTA GACCCGGGTA GGCGGACTAT GACTTAGTTG C 31

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

GAATTCGCTA GCTACGTACC CACCCTCTGC TGCCCCCAAC 30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:

AGATCTTCTA GACCCGGGCT AAGATGCCTT CTCTCTCCAT C 31

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6:

GAATTCGTCA GCTACGTAAC AATGCTCAGG GTGTCAAAGC A 31

CLAIMS:

1. A process which comprises the positioning, within a living cell, of a target molecule and an inhibitor for said target molecule, said positioning being such that the concentration of the inhibitor molecule with respect to the target molecule is enhanced.

2. The claim 1 process in which the target molecule is an RNA molecule and the inhibitor is a ribozyme.

3. The claim 1 process in which the target molecule is an HIV-1 RNA molecule and the inhibitor is a ribozyme which cleaves said HIV-1 RNA molecule.

4. The method which comprises co-localizing a target molecule and an inhibitor for said target molecule within a living cell.

5. A living cell in which a target molecule and an inhibitor for said target molecule are co-localized.

6. The claim 4 method in which the target molecule is an RNA molecule and the inhibitor is a ribozyme which cleaves said RNA molecule.

7. The living cell of claim 5 in which the target molecule is an RNA molecule and the inhibitor is a ribozyme which cleaves said RNA molecule.

8. A method which comprises co-localizing within a living mammalian cell

an RNA target molecule, and

a ribozyme which cleaves said RNA target molecule

said ribozyme including

(i) the dimerization or packaging signal of said RNA target molecule, or

(ii) a sequence capable of pairing with said RNA molecule at a site upstream of the primer binding site to the 3′ end of $tRNA^{LYS3}$, or

(iii) a 3′ untranslated region (UTR) of said RNA molecule.

9. The claim 8 method in which said RNA target molecule is an HIV-I RNA molecule.

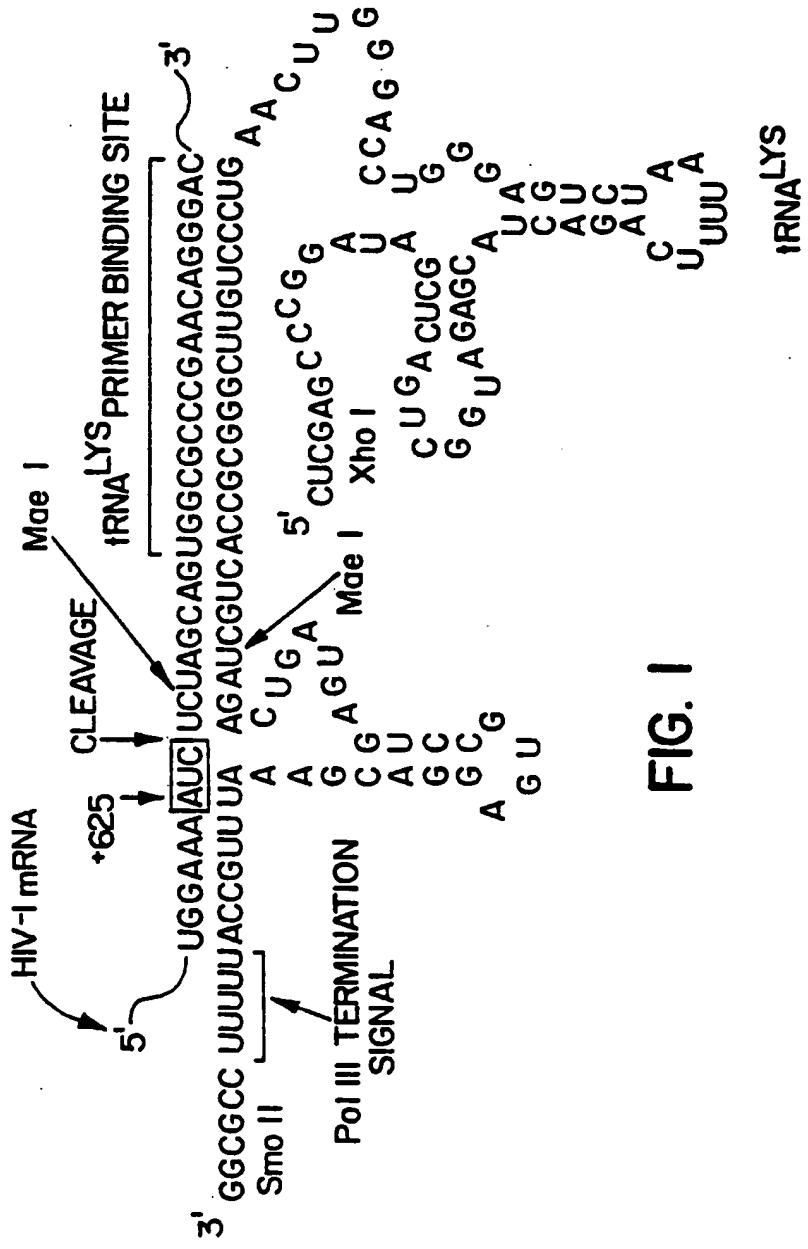
HIV-1 mRNA
5'
UGGAAAAUCUUUUUACCGUUUA
3'
GGCGCC
Smo II
+625
CLEAVAGE
Mae I
tRNALYS PRIMER BINDING SITE
AGAUCGGUCACCGCGGGCUUGUCCCUG
Pol III TERMINATION SIGNAL
Mae I
5'
CUCGAG
Xho I
3'
AACUU
CCAGGG
UGGG
AUA
CGU
AUGC
AUA
CUUU
A
tRNALYS

FIG. I

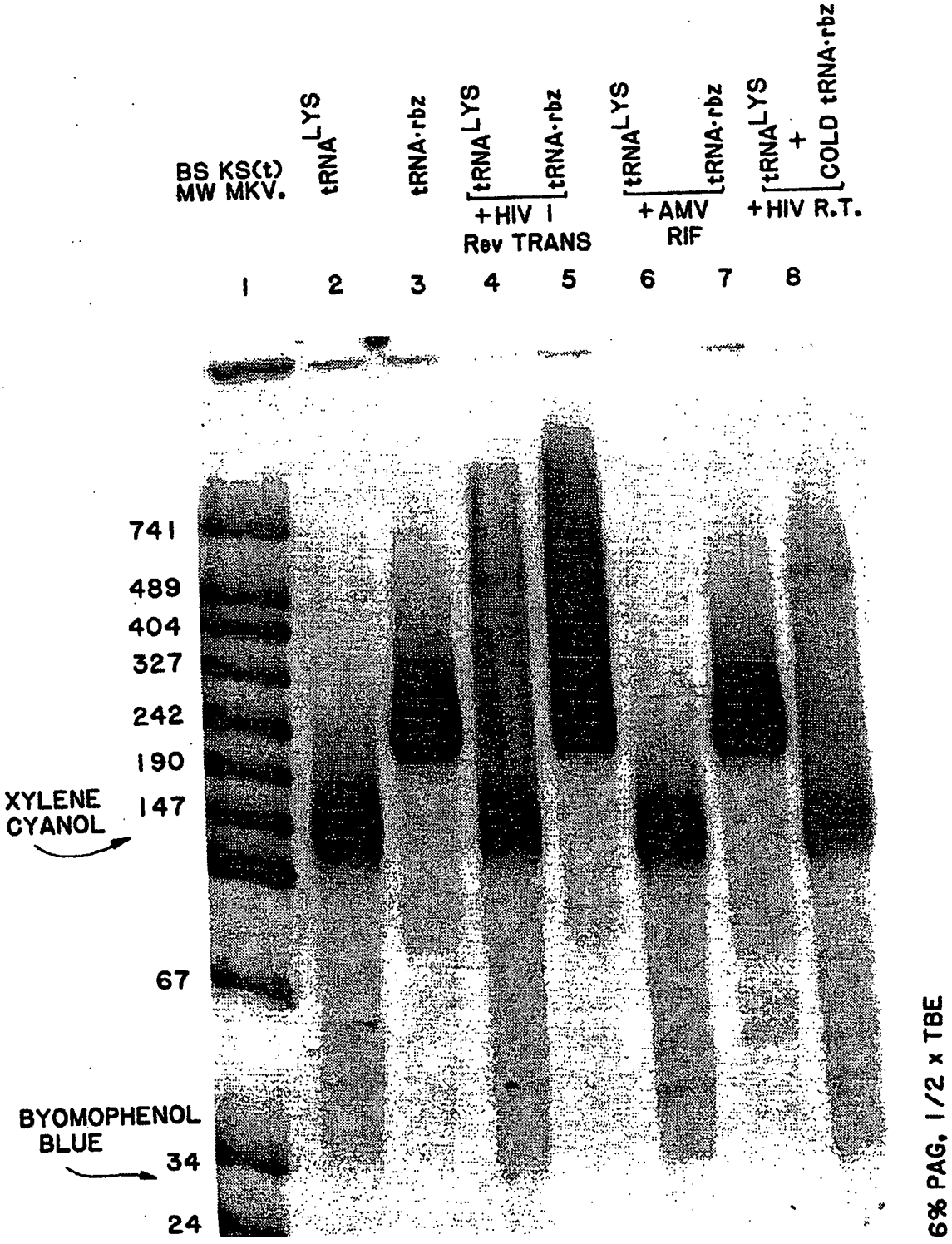
BS KS(t) MW MKV.
tRNA^{LYS}
tRNA·rbz
[tRNA^{LYS} tRNA·rbz] +HIV I Rev TRANS
[tRNA^{LYS} tRNA·rbz] +AMV RIF
[tRNA^{LYS} + COLD tRNA·rbz] +HIV R.T.
1 2 3 4 5 6 7 8
741
489
404
327
242
190
147
67
34
24
XYLENE CYANOL
BYOMOPHENOL BLUE
6% PAG, 1/2 x TBE

FIG. 2

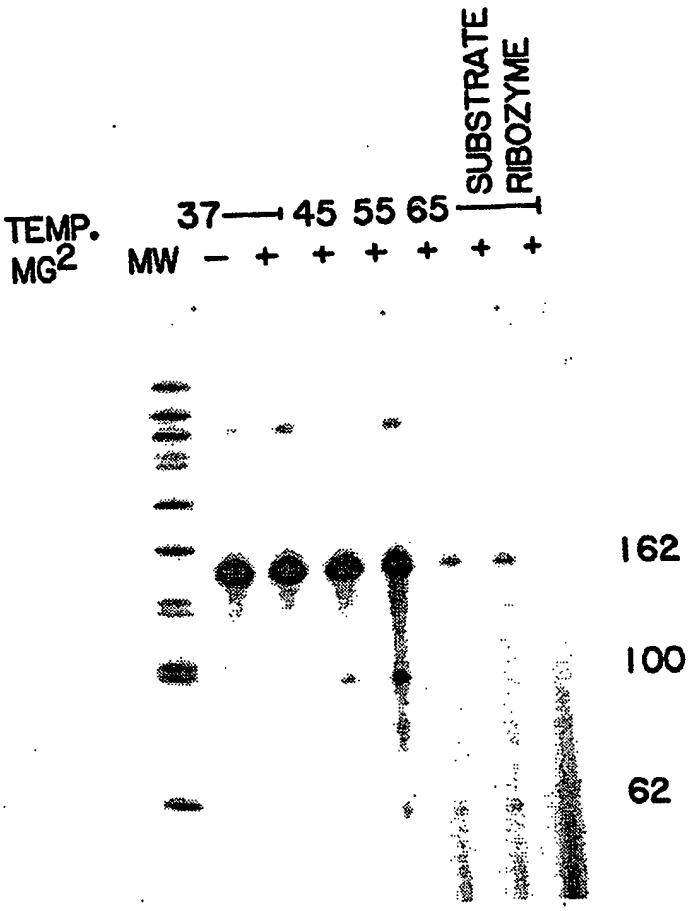

FIG. 3

FIG. 4

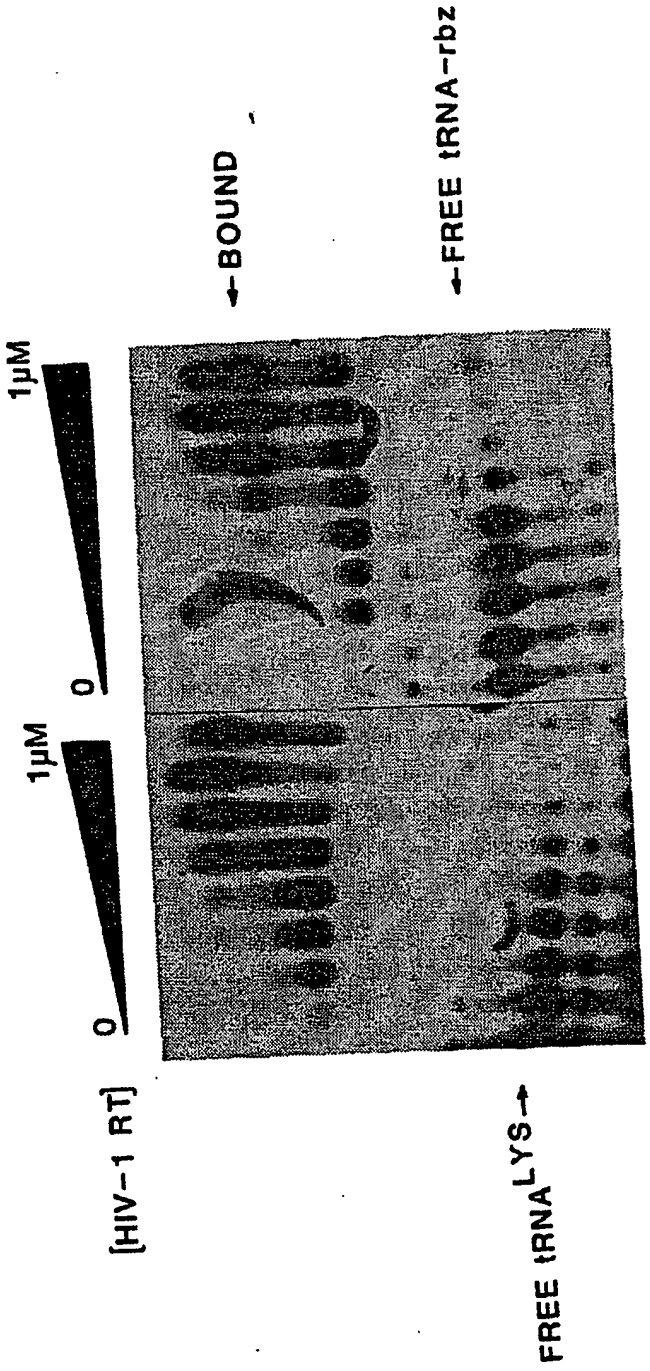
←BOUND
←FREE tRNA-rbz
1μM
0
1μM
0
[HIV-1 RT]
FREE tRNA LYS→
tRNA LYS
tRNA-Rbz

FIG. 5A

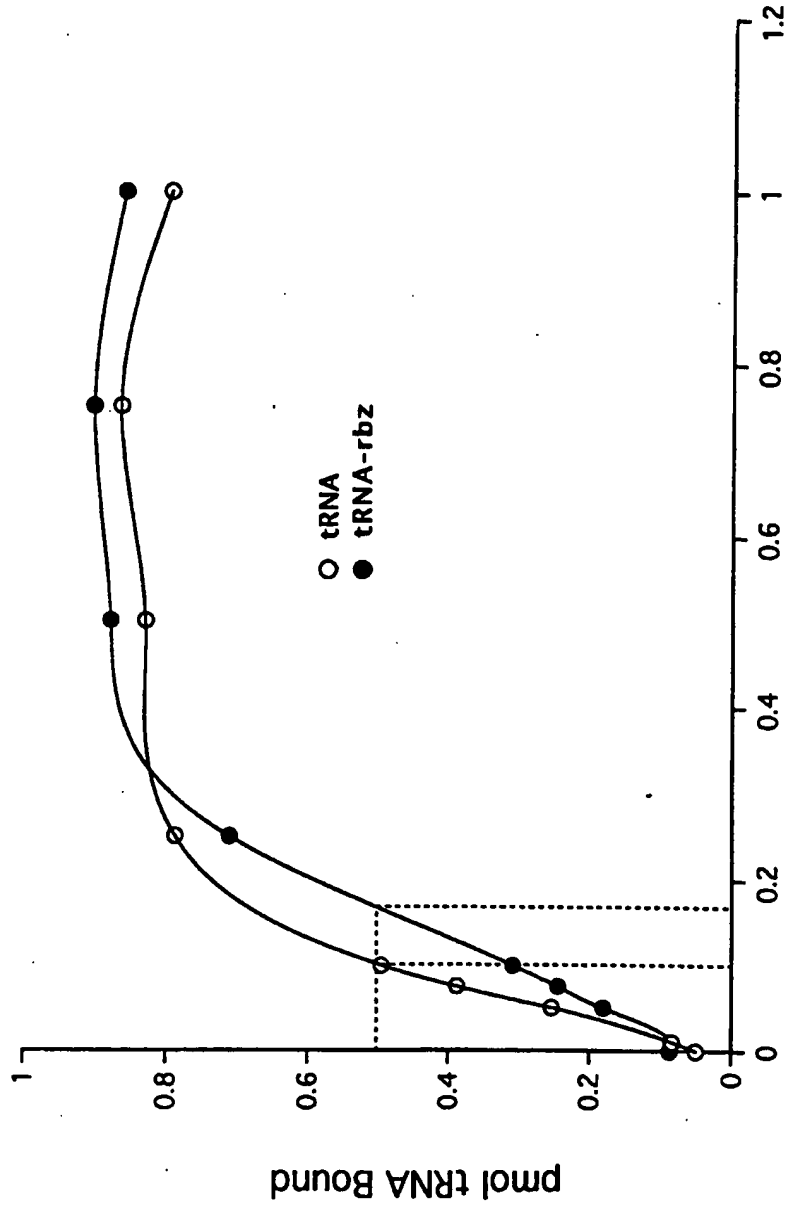
pmol tRNA Bound
0
0.2
0.4
0.6
0.8
1
0
0.2
0.4
0.6
0.8
1
1.2
[RT] (μM)
tRNA
tRNA-rbz

FIG. 5A-1

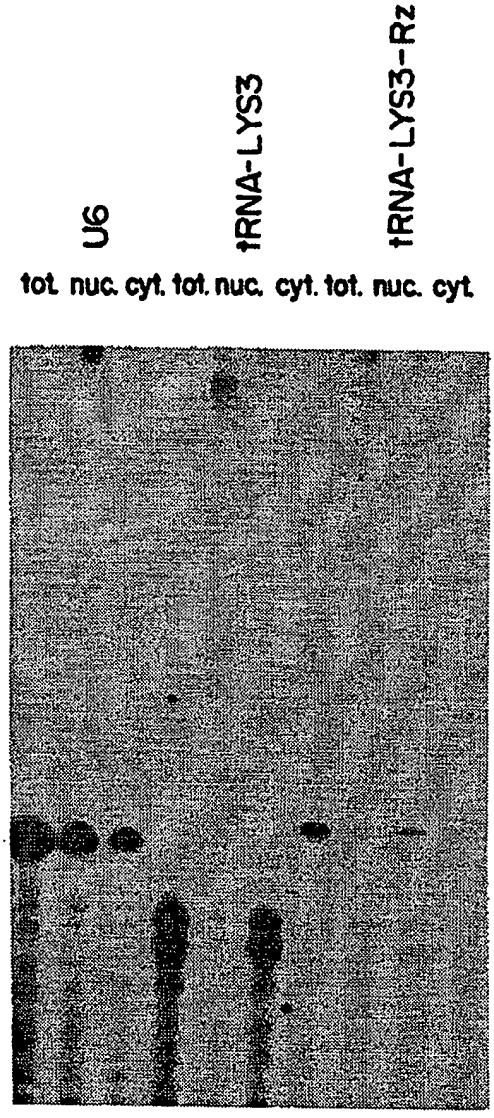

FIG. 5B

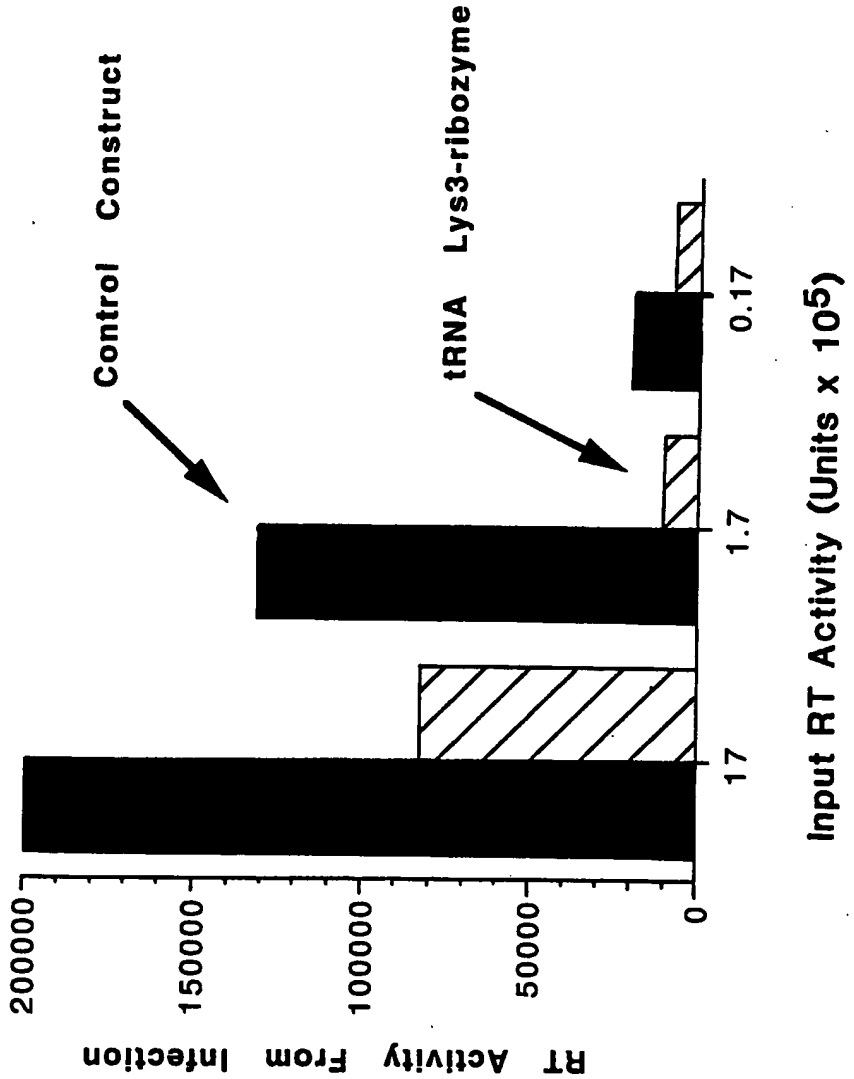
Control Construct
tRNA Lys3-ribozyme
RT Activity From Infection
200000
150000
100000
50000
0
17
1.7
0.17
Input RT Activity (Units x 10^5)

FIG. 5C-1

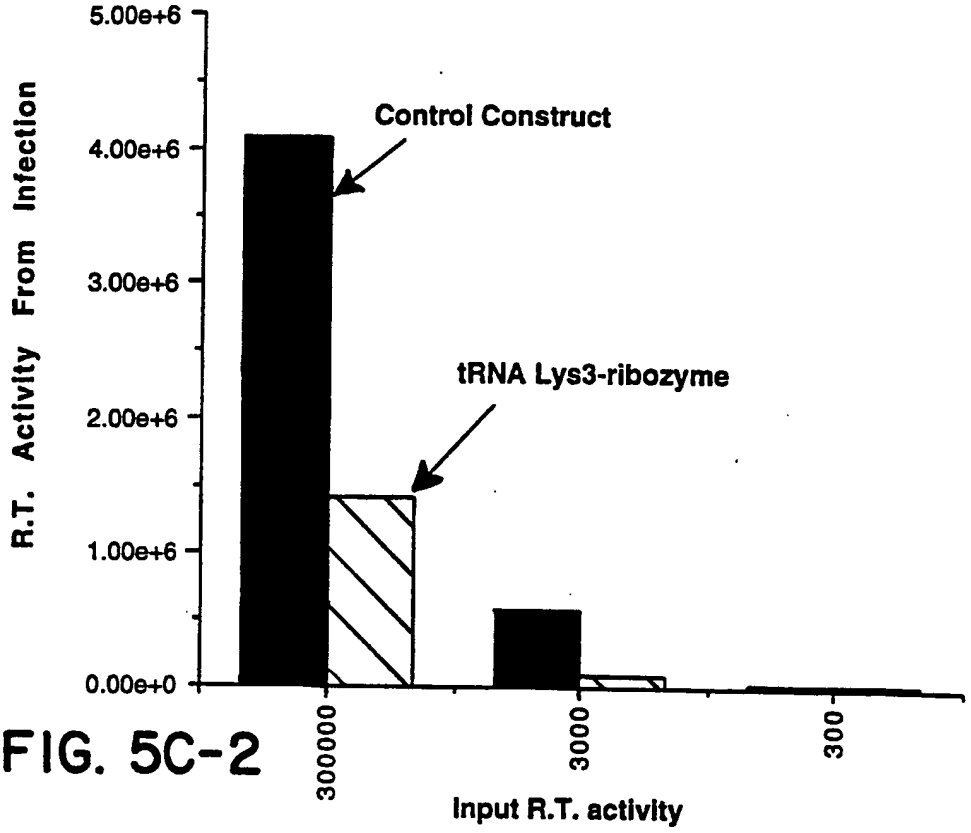

FIG. 5C-2

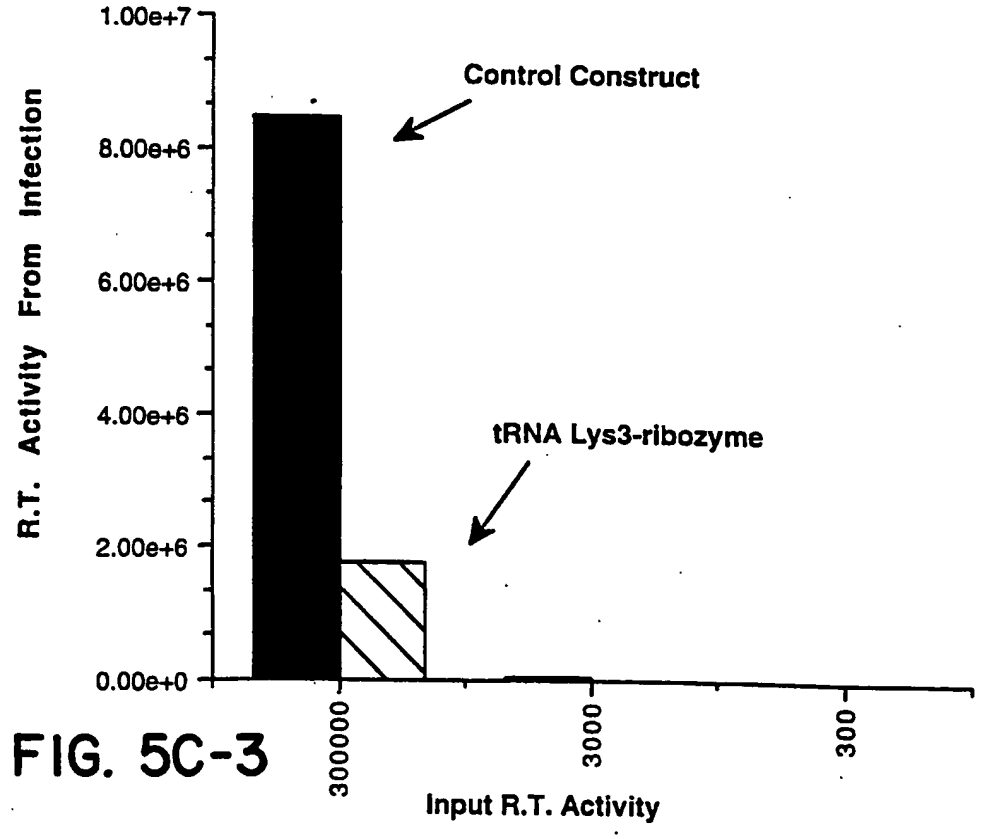

FIG. 5C-3

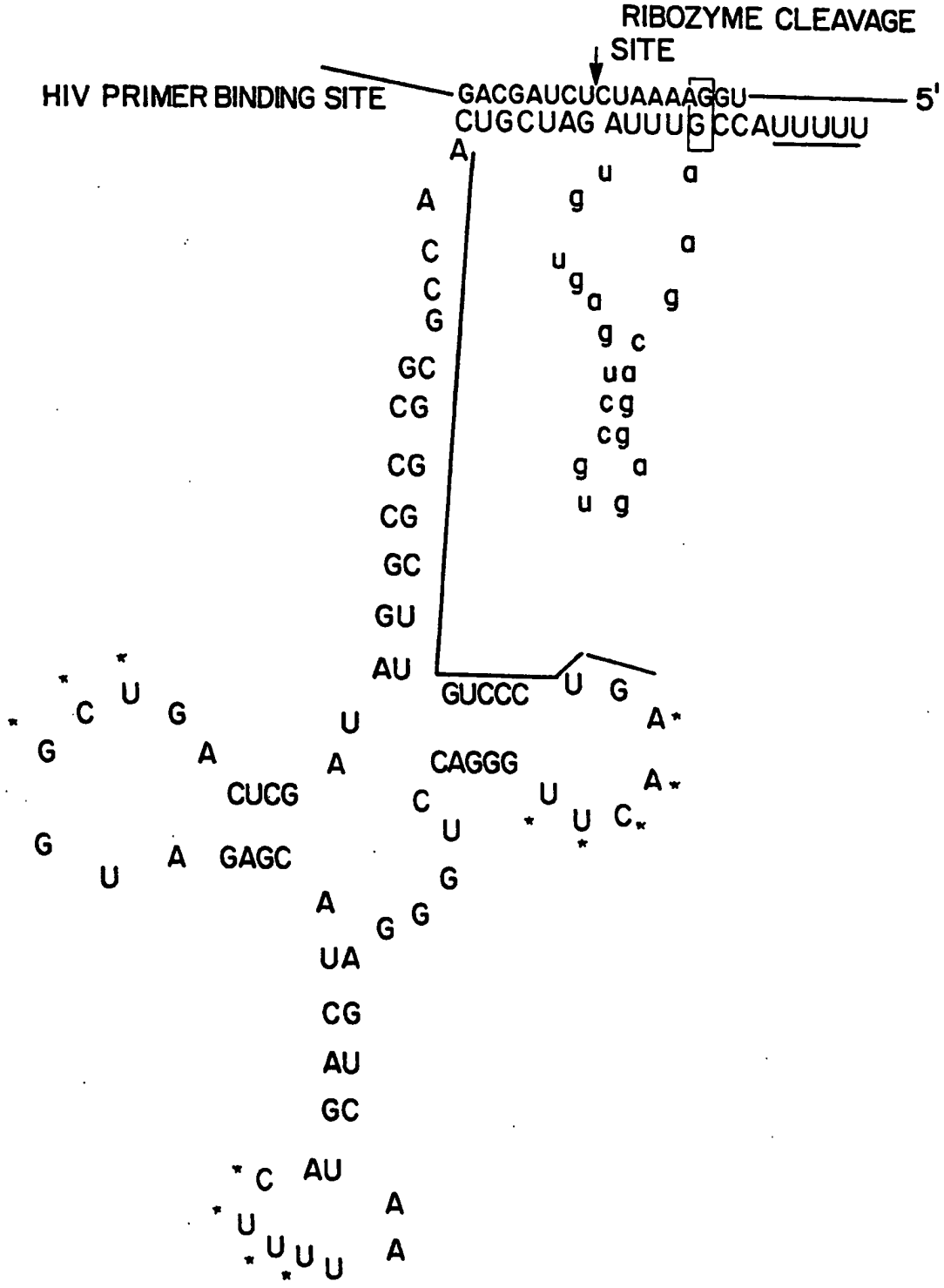
RIBOZYME CLEAVAGE SITE
HIV PRIMER BINDING SITE
GACGAUCUCUAAAAGGU
5'
CUGCUAG AUUUGCCAUUUUU
GUCCC
CAGGG
CUCG
GAGC

FIG. 6

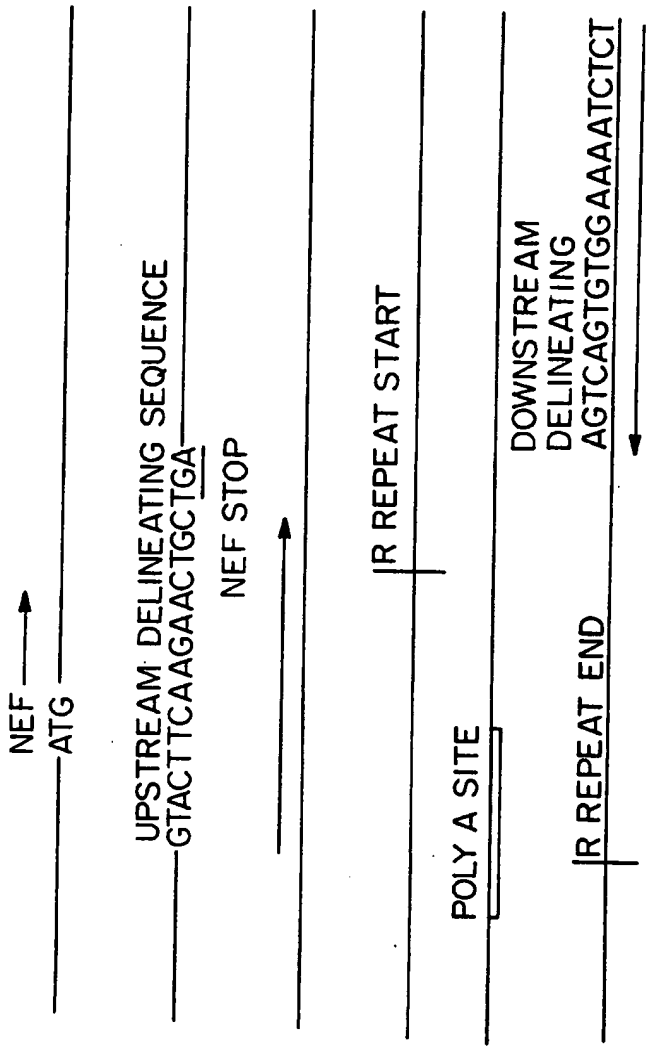
NEF
ATG
UPSTREAM DELINEATING SEQUENCE
GTACTTCAAGAACTGCTGA
NEF STOP
IR REPEAT START
POLY A SITE
DOWNSTREAM
DELINEATING
AGTCAGTGTGGAAAATCTCT
IR REPEAT END

FIG. 7

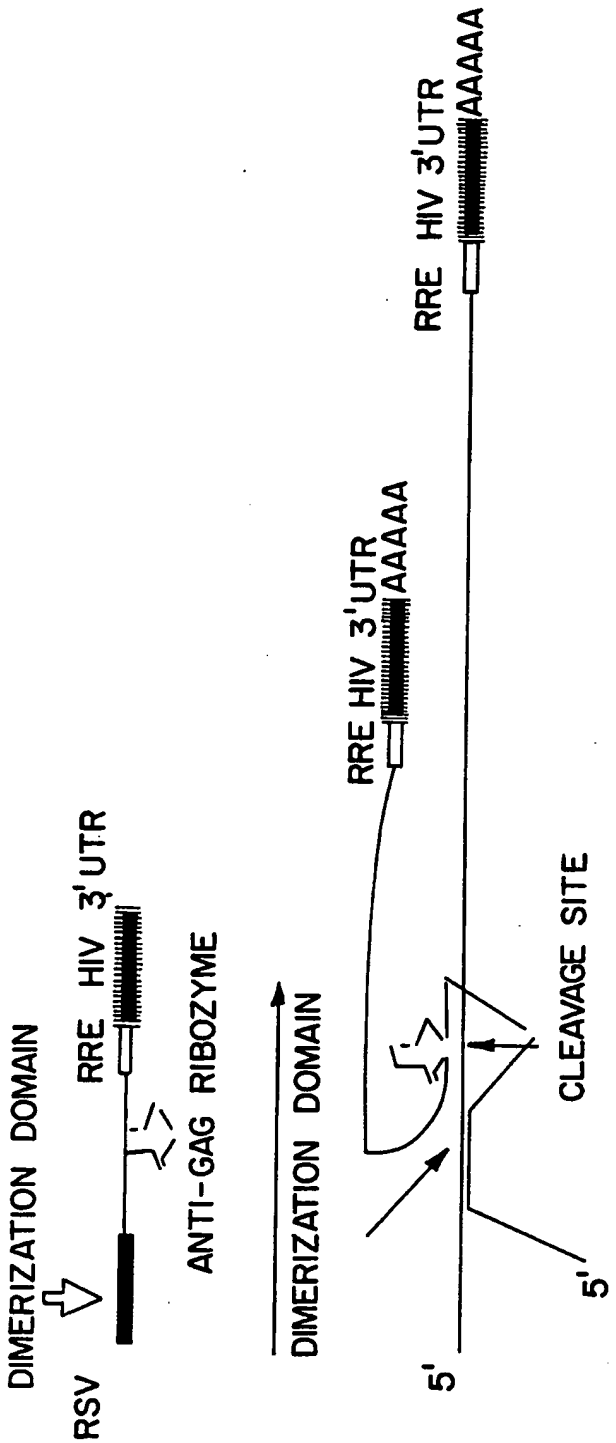
DIMERIZATION DOMAIN
RSV
RRE HIV 3'UTR
ANTI-GAG RIBOZYME
DIMERIZATION DOMAIN
RRE HIV 3'UTR
AAAAA
RRE HIV 3'UTR
AAAAA
CLEAVAGE SITE
5'
5'

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13798

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/43, 47/48; C12N 9/22, 5/22; C12Q 1/25
US CL :435/6,91.31,240.2; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6,91.31,240.2; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog: BIOSIS, MEDLINE, Derwent Biotechnology Abstracts, CAS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Journal of Biological Chemistry, Volume 269, Number 20, issued 20 May 1994, Arts et al., "Comparision of deoxynucleotide and tRNA Lys-3 as primers in an endogenous human immunodeficiency virus-1 in vitro reverse transcription/template-switching reaction", pages 14672-14680, see entire document.	1-9
X -- Y	Biochemical and Biophysical Research Communications, Volume 192, Number 2, issued 30 April 1993, Wisotzkey et al., "Cleavage of cottontail rabbit papillomavirus E7 RNA with an anti-E7 ribozyme", pages 833-839, see entire document.	1 ----- 2-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
05 FEBRUARY 1995

Date of mailing of the international search report
09 MAR 1995

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US94/13798

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 247, issued 09 March 1990, Sarver et al., "Ribozymes as potential anti-HIV-1 therapeutic agents", pages 1222-1225, see entire document.	2-7

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(60) Parent Application or Grant
(63) Related by Continuation
US 08/185,827 (CIP)
Filed on 24 January 1994 (24.01.94)

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: INHIBITORS AND TARGET MOLECULE CO-LOCALIZATION

(57) Abstract

The invention provides mechanisms for the co-localization in a living cell of a target molecule and of an inhibitor for the target molecule. The invention also provides novel chimeric $tRNA^{LYS}$-ribozyme molecules that compete effectiely with $tRNA^{LYS}$ for HIV-1 reverse transcriptase binding sites. The chimeric human $tRNA^{LYS}$-ribozymes inhibit reverse HIV transcription by delivering inhibitors such as ribozymes of HIV-1 reverse transcriptase directly to the virion particle and render it non-functional. The chimeric molecules of the invention thus serve as highly specific non-toxic therapeutic agents and vaccines for viral, including lentiviral, infections. These chimeric molecules also reveal a novel, site specific RNA cleaving activity of HIV-1.

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INHIBITORS AND TARGET MOLECULE CO-LOCALIZATION

This invention was made with government support under Grant No. AT 25959 awarded by the National Institutes of Health. The government has certain rights in the invention.

This application is a continuation-in-part of Serial No. 08/185,827 filed January 24, 1994.

FIELD OF INVENTION

This invention relates to mechanisms for bringing two or more molecules together in a living cell. More particularly, the invention relates to mechanisms for bringing together with a cell a target molecule and an inhibitor therefore in a manner effective to increase the concentration of the inhibitor with respect to the target. For example, the invention relates to mechanisms for increasing the cellular concentration of a ribozyme with respect to a target mRNA molecule to be cleaved by the ribozyme.

One embodiment of this invention relates to chimeric $tRNA^{LYS}$ ribozyme molecules which compete effectively with $tRNA^{LYS}$ for binding to HIV-1 reverse transcriptase. These chimeric molecules provide a co-localization mechanism for delivering inhibitors of HIV-1 and reverse transcriptase to the virion particle itself.

BACKGROUND OF THE INVENTION

RNA is unusual in its ability both to store information in its nucleotide sequence and to function as an enzymatic catalyst of specific reactions (1,2). This combination of attributes has created opportunities for engineering RNA enzymes (ribozymes) which can be used to cleave and functionally inactivate targeted RNAs. Some of the attributes of ribozymes which make them attractive candidates for therapeutic agents are their ability to site-specifically cleave targeted RNAs, cleave multiple substrates, and their ability to be engineered for improved cleavage specificity and enhanced catalytic turnover (3,4). There are five catalytic motifs which have been successfully modified and/or adapted for use in

ribozyme applications. These are the group I introns, RNAse P, the hammerhead and hairpin motifs, and the self-cleaving domain of the hepatitis delta virus (5,3,6,7). Each of these engineered ribozymes only requires a divalent metal cation for activity (usually MG^{++}) which participates in the chemistry of the cleavage reaction (8,9,10).

The therapeutic use of ribozymes is an attractive goal which merges the basic and applied sciences. Since all genes are expressed through RNA intermediates, the potential applications are primarily limited by knowledge of the disease or disease associated with a given RNA. In the case of viral infections, such as HIV, ribozymes can be tailor made to cleave viral transcripts, thereby leaving cellular transcripts untouched. Because of this, HIV is a prime target for ribozyme inactivation. This concept was successfully tested by intracellularly expressing a hammerhead ribozyme targeted to a gag cleavage site, which resulted in up to a 40 fold reduction in viral p24 antigen production in HeLa CD4+ cells challenged with HIV (11,12). As a retrovirus with an RNA genome, there are hundreds of potential ribozyme cleavage sites along the length of the viral genomic and subgenomic RNAs. Since the virus mutates rapidly, and can become resistant to most drugs developed to inhibit a single viral target (13), ribozymes have become an important alternative for anti-viral therapeutic agents since multiple ribozymes targeted to a number of different sites can be simultaneously delivered to cells for inhibition of HIV (14). There are two times in the viral life-cycle when ribozymes could be effective against HIV infection. The first immediately following infection prior to proviral DNA formation, when all or part of the viral genome is still in the form of RNA, and the second following the establishment of integrated provirus from which spliced and a full length viral

transcripts are produced (15,16). An important consideration is the observation that HIV can infect quiescent T-lymphocytes, wherein proviral DNA synthesis is initiated but is incompletely reverse transcribed (17). If a ribozyme is present in the infected cell cytoplasm, it theoretically could protect cells from permanent infection by cleaving the RNA at this early step, before the T-cell becomes activated. In support of this type of action by a ribozyme, Yamada, et al., (18) have demonstrated a 50-100 fold reduction in HIV proviral DNA formation in cells expressing a hairpin ribozyme targeted to a site in the 5′ leader sequence.

A number of reports demonstrating varying levels of ribozyme mediated protection of cultured cells from HIV infection have been published (14,19,20,21,22,12,23,24). The most encouraging results of ribozyme mediated inhibition of HIV utilized a hairpin ribozyme targeted to a highly conserved GUC cleavage site in HIV (22,18,24). Expression of this ribozyme gave rise to long term resistance to infection, including resistance to a variety of HIV isolates. Studies recently completed demonstrate that hammerhead ribozymes targeted to conserved sites in the tat and a shared tat-rev exon, when expressed from a Moloney viral vector LTR can confer protection to cells in culture for at least 21 days (Gene 149:33-39 (1994), incorporated herein by reference). Despite these reported successes, the observation has been made that ribozyme mediated protection of cells can be overcome with increasing multiplicity of infection, and in some instances with prolonged culture items. In a patient setting, this is likely to be a serious problem since there is substantial evidence suggesting that the virus is highly concentrated in the lymphoid system, providing in essence, a high multiplicity of infection to CD4+ cells entering that environment (25,26,27). A somewhat different problem is that of the genetic

variability of HIV (15). Although it has not been formally demonstrated in experimental models, it is reasonable to assume that viral resistance to ribozymes can and will occur, especially in a patient setting, where the pool of viruses is bound to be genetically heterogeneous (28,13,29,30).

The first steps in solving this problem involve developing a detailed understanding of how ribozymes can be made to function more effectively in an intracellular environment. For most RNAs, very little is known about the mechanisms regulating the pathway of movement from transcription through translation, and in the case of HIV, from transcription through packaging. There is increasing evidence, although some of it still controversial, that nuclear transcripts are processed and migrate along specific tracks, which predicts non uniform distributions of specific nuclear transcripts (31). Following export from the nucleus, there is also increasing evidence that a variety of RNAs can be specifically localized within the cytoplasm as well (32). From the prospective of ribozyme therapeutic applications, capitalizing upon the localization properties of RNAs could facilitate intracellular functioning of ribozymes by allowing them to co-localize with their target RNAs. Sullenger and Cech (1993) (33) (incorporated herein by reference) have directly tested this idea by utilizing the dimerization and packaging signal of a Moloney murine leukemia virus genomic RNA to co-localize a hammerhead ribozyme with its target, the lac Z gene carried by another recombinant Moloney viral vector. They found that up to 90% inhibition of infective virus production could be achieved as a result of co-packaging the ribozyme and the lacZ target containing viral RNAs. Their data showed that inhibition of lacZ expression was only achieved when the ribozyme was co-packaged with the genomic target RNA. Thus, mRNAs

harboring the lacZ sequence, but lacking the packaging signal, were not destroyed by the ribozyme, clearly demonstrating the usefulness of a co-localization strategy for ribozymes.

A different co-packaging strategy which takes advantage of the fact that HIV utilizes $TRNA^{LYS3}$ as a site specific primer for reverse transcription is described in U.S. Serial No. 08/185,827. A ribozyme capable of pairing with and cleaving HIV at a site just upstream of the primer binding site to the 3′ end of $tRNA^{LYS3}$. The strategy in that application is that the chimeric molecules could be bound by HIV reverse transcriptase, and captured during viral assembly.

During the series of events that RNAs undergo from their birth to their death, they are constantly associated with proteins (34). It is a virtual certainty that ribozymes will encounter proteins in an intracellular environment which will have an effect, either positive or negative, on their activity. Tsuchihashi, et al. (1993) (35), Herschlag, et al., (1994) (36) and Bertrand and Rossi (1994) (37) have observed RNA binding proteins such as HIV-1 encoded NCp7 and cellular hnRNP A1 can facilitate ribozyme catalytic turnover in vitro.

The ribozyme-target co-localization strategy described in Serial No. 08/185,827 involves utilizing the $tRNA^{LYS3}$ primer for reverse transcriptase (RT) as a vehicle for co-localizing a ribozyme with HIV genomic RNA, and potentially into the virion itself. The strategy is based upon the well established interactions of HIV RT with cellular $tRNA^{LYS3}$, which is the primer tRNA used by all the mammalian lentiviruses. This tRNA is selectively bound by RT, and in the presence of the nucleocapsid protein NCp15 (or NCp7), unwinds the aminoacyl stem of the tRNA, allowing it to base pair with the viral PBS (38). The premise of Serial No. 08/185,827 is that a

ribozyme appended to the 3′ terminus of $tRNA^{LYS3}$ could be captured by RT, co-packaged with the virus, and the ribozyme would be aligned to cleave the viral genomic RNA and destroy its infectivity. Available data supports the hypothesis. These data are summarized as follows: (1) The tRNA-ribozyme binds selectively to HIV RT with a binding affinity virtually identical to a synthetic $tRNA^{LYS3}$. (2) The tRNA-ribozyme is expressed as a Pol III transcript when transfected into 293 cells, and the ribozyme moiety is not processed from the transcript, although the 5′ precursor segment of the tRNA-ribozyme is processed normally. By including the CCA in the transcripts, which is normally added post-transcriptionally to the tRNA, these molecules are not subject to the normal 3′ processing events. (3) The tRNA-ribozyme is exported to the cytoplasm, making it available for binding with RT. (4) When the tRNA ribozyme is transiently transfected into 293 cells, there are equivalent levels of tRNA-ribozyme transcript to endogenous $tRNA^{LYS3}$. (5) Co-transfection of the tRNA-ribozyme gene with pNL4-3 DNA into 293 cells resulted in a 4 to 12 fold reduction in infectious virus production relative to control constructs. See Figure 5.

It has been demonstrated that the entire $tRNA^{LYS}$ molecule as well as various segments of the tRNA per se are capable specifically of interacting with HIV-1 transcriptase. See Barat, et al. EMBO Journal 8:3279-3285 (1989); Khan, et al. J. Bio. Chem 267:6689-6695 (1992); Weiss, et al., Gene 111:183-197 (1992). Ben-Artzi, Proc. Natl. Acad. Sci. USA 89:927-931 (1992) reports an RNAse cleavage activity associated with HIV-1 reverse transcriptase. This activity is shown to cleave only HIV-1 RNA, not the primer.

Prior to this invention there has been no report of chimeric $tRNA^{LYS}$-ribozyme molecules.

DEFINITION

Co-localization: As used in this application, the term co-localization means the positioning of two or more molecules within a living cell, one of which is a target and the other an inhibitor of the target that the concentration of the inhibitor with respect to the target is increased within the cell and function or expression of the target is constrained or inhibited.

Co-localization may be accomplished by covalent linkage (cis-ribozyme) or via co-targeting the viral capsid. A specific embodiment of co-localization pursuant to this invention entails the positioning within a living mammalian cell of a ribozyme adjacent a virion particle to cleave virion RNA.

SUMMARY OF THE INVENTION

This invention provides co-localization mechanisms and living cells in which an inhibitor and a target are co-localized by such mechanisms. An important object of the invention is to provide novel intracellular immunogens for vaccines against viral infections.

One preferred embodiment of this invention provides novel chimeric $tRNA^{LYS}$-ribozyme molecules that compete effectively with $tRNA^{LYS}$ for HIV-1 reverse transcriptase binding sites. The chimeric human $tRNA^{LYS}$-ribozymes inhibit reverse HIV transcription by delivering inhibitors such as ribozymes of HIV-1 reverse transcriptase directly to the virion particle and render it non-functional. The chimeric molecules of this invention thus serve as highly specific non-toxic therapeutic agents.

These chimeric molecules also reveal a novel, site specific RNA cleaving activity of HIV-1.

DESCRIPTION OF THE FIGURES

Figure 1 (SEQ ID NO. 1 and SEQ ID NO. 2) shows the structure of one chimeric ribozyme. This $tRNA^{LYS}$-ribozyme construct has been cloned into a Blue Script

transcription vector using SacII and XhoI restriction sites. Following linearization at the SacII site the chimeric RNA can be transcribed in vitro using bacteriphase T-7 RNA polymerase. There is also a Mae I restriction site in between the tRNA and ribozyme moieties, allowing the tRNA to be transcribed independently of the ribozyme.

Figure 2. This gel shift experiment shows binding of the chimeric $tRNA^{LYS}$-ribozyme to HIV-1 reverse transcriptase. The eight lanes of the gel from left to right are:

1. Molecular weight marker.

2. $tRNA^{LYS}$ in vitro transcript which has extra bases at both the 5′ and 3′ ends. The extra 5′ bases are from the Blue Script poly linker between the T-7 promoter and the XhoI site. There are six extra nucleotides at the 3′ derived from the nucleotides after the CCA of the tRNA to the Mae I site which separates the tRNA from the ribozyme.

3. $tRNA^{LYS}$-ribozyme in vitro transcript which has the same extra 5′ bases as $tRNA^{LYS}$, but terminates at SacII site at the end of the ribozyme moiety.

4. $tRNA^{LYS}$-transcript incubated with HIV-1 reverse transcriptase.

5. $tRNA^{LYS}$-ribozyme transcript incubated with HIV-1 reverse transcriptase.

6. $tRNA^{LYS}$-transcript incubated with AMV reverse transcriptase.

7. $tRNA^{LYS}$-ribozyme incubated with AMV reverse transcriptase.

8. $tRNA^{LYS}$ with competing, non-radioactively labelled tRNALYS-ribozyme incubated with HIV-1 reverse transcriptase.

This Figure 2 shows that the chimeric $tRNA^{LYS}$-ribozyme specifically binds to HIV-1 reverse transcriptase by a shift in radioactivity when HIV-1

reverse transcriptase is present. Cold $tRNA^{LYS}$-ribozyme competes with $tRNA^{LYS}$ for binding to HIV-1 reverse transcriptase as indicated by the reduced radioactive shift in lane 8.

Figure 3. This experiment demonstrates cleavage of a 162 nucleotide, radioactively labelled HIV-1 RNA containing the primer binding site plus sequences upstream of this and including the AUC cleavage signal for the ribozyme. The cleavage products are 101 and 61 bases. The extent of cleavage increases with increasing temperature.

Figure 4. Demonstration of the novel RNAse activity of HIV-1 reverse transcriptase when $tRNA^{LYS}$-ribozyme and HIV-1 primer binding site transcripts are incubated together in the presence of HIV-1 reverse transcriptase. The $tRNA^{LYS}$-ribozyme is radioactively labelled, and the HIV-1 RNA is non-radioactive. The cleavage products result in the tRNA moiety being separated from the ribozyme moiety. This result also demonstrates that the chimeric $tRNA^{LYS}$-ribozyme cannot serve as a primer for HIV-1 reverse transcriptase.

The lanes are, left to right: $tRNA^{LYS}$-ribozyme alone, $tRNA^{LYS}$-ribozyme plus HIV-1 reverse transcriptase, no deoxyribonucleoside triphosphates; $tRNA^{LYS}$-ribozyme plus HIV-1 reverse transcriptase plus deoxyribonucleoside triphosphates; last two lanes same as lane 3 except lane 4 has AMV reverse transcriptase and lane 5 has MLV reverse transcriptase. The black dots mark the HIV-1 reverse transcriptase cleavage products. Unlabelled HIV-1 primer binding site containing 162 nucleotide transcript was present in each lane. None of the reverse transcriptases can utilize the $tRNA^{LYS}$-ribozyme as a primer since it has 12 nucleotides at the 3' end which cannot base pair with the HIV-1 primer binding site RNA.

Figure 5. Illustrates A: RT binding to $tRNA^{LYS3}$-ribozyme. B: Primer extension analyses demonstrating

nuclear localization of chimeric transcript. The primer for the tRNA-ribozyme is in the ribozyme moiety, and the primer for $tRNA^{LYS3}$ is at the 3' end of the tRNA.
C: Results of infectious virus assays carried out with supernatents from 293 cells transfected with tRNA-ribozyme or control construct (ribozyme minus tRNA in same vector) and co-transfected with pNL4-3. Three independent experiments are presented.

Figure 6 illustrates the $tRNA^{Lys3}$-ribozyme which is the starting molecule. The asterisks indicate sites which UV crosslink to HIV RT or are protected from RNAse digestion in the presence of RT. A deliberately created mismatch in the ribozyme pairing arm is indicated with a boxed in nucleotide pair. This was done to eliminate a stretch of 4T's in the ribozyme gene which could serve as a Pol III termination site. The authentic termination site (5 U's or T's in DAN) is underlined. The T loop-stem and aminoacyl acceptor stem which pair with the HIV primer binding site are overlain with a heavy line.

Figure 7 is a schematic representation of nef and 3'UTR region to be included in ribozyme and GH reporter systems. The delineating sequences are the extremities of the DNA amplified by PCR. These sequences are from the pNL4-3 proviral clone and encompass the region of nucleotides 9389 through 9704.

Figure 8 represents a construct containing anti-HIV-1 ribozyme expressed in context of dimerization domain and RRE to facilitate co-localization with HIV full-length genomic RNAs.

GENERAL DESCRIPTION OF THE INVENTION

The invention provides various co-localization mechanisms. These mechanisms include, among others, (i) utilization of specific RNA trafficking pathways to both the target and the inhibitor, (ii) utilization of protein interaction with inhibitor and target molecules, e.g., HIV-1 RT (see Sullenger and Cech (33)), (iii) use

of cellular proteins which subcellularly compartmentalize the inhibitor to the target or a specific target site; (iv) use of cis-acting sequence substituents on ribozyme transcripts to direct the ribozyme to a specific subcellular trafficking pattern or site; (v) ribozymes which include any molecule or moiety that specifies a distinct intracellular trafficking pattern and target localization site.

1. Co-Localization of Ribozymes with HIV-1 or Cellular RNA Targets.

a. $tRNA^{LYS3}$-ribozyme chimeric molecules

One of the most important problems facing the routine use of ribozymes as therapeutic agents is that of maximizing effective interactions of ribozymes and target RNAs. It has been convincingly demonstrated by Sullenger and Cech (33) that co-localization of a ribozyme and target RNA through a retroviral packaging signal can dramatically enhance the effectiveness of the ribozyme pairing with, and cleaving its substrate. As noted, Serial No. 08/185,827 describes somewhat different co-localization strategy with the $tRNA^{LYS3}$-ribozyme chimeras (see Progress Report section), which are bound by HIV reverse transcriptase allowing alignment of the ribozyme during packaging of the virus. This approach has been successful and has led to a reduction of infective viral titer as a consequence of co-expressing chimeric tRNA-ribozymes with HIV proviral DNA. In order to make this a more generally useful strategy, it is useful to develop chimeric molecules which effectively compete against cellular tRNAs for binding to RT, yet do not create a general toxicity problem. One of the goals of this invention is to develop genetic variants of $tRNA^{LYS3}$ which maintain the sequence and structural features required for interaction with a ribozyme for cleavage, yet are dissimilar enough from cellular $tRNA^{LYS3}$ so as not to interfere with normal cellular metabolism. The use of

these variants will also be coupled with enhanced intracellular expression systems. The identification of molecules which can still interact with the primer binding site of HIV (which means leaving at least the 3' segment of the amino-acyl acceptor stem intact), thereby allowing alignment of a ribozyme (appended to the 3' end) with a cleavage site adjacent to the viral primer binding site is contemplated.

Since high levels of expression of the $tRNA^{LYS3}$-ribozyme chimeric gene during transient transfection were observed, it is reasonable that inserting multiple, tandem copies of the tRNA ribozyme chimeric genes in a vector such as adeno associated virus (AAV) can also lead to high level expression.

A potential strategy for increasing the intracellular levels of the chimeric ribozyme transcript is to express them from heterologous promoters. For those variants which lack the A or B boxes, this will be a necessity. For variants which have maintained these elements, site directed changes which eliminate the promoter function will allow testing of these constructs using heterologous promoters. Several candidate promoters have been developed for ribozyme expression. The human U6 snRNA gene has a Pol III promoter element which is 5' of the coding sequence (Parry, et al. (39)). Transcription terminates after a string of 5 Uracil residues, resulting in a RNA with well defined ends. It has been demonstrated that this promoter can be used to transcript ribozyme containing RNAs which localize to the cytoplasm. A potential advantage of this promoter is that transcription can be engineered to initiate at the +1 sequence of the tRNA molecule, thus eliminating any need for processing a 5' leader, and allowing the synthesis of a very defined transcript.

b. The 3' untranslated region (UTR) as an RNA trafficking signal-model for ribozyme-target co-localization.

The factors which dictate the trafficking and intracellular localization of RNAs are poorly understood. There are some reports which suggest that RNAs may "track" along specific paths following transcription and transport to the cytoplasm (reviewed in 31). There are numerous examples of messenger RNAs which localize to specific regions of the cytoplasm as well. The most well studied localized RNAs are the oocyte and early embryo mRNAs of Drosophila and Xenopus (32). Other mRNAs such as actin have been shown to localize to cytoskeletal components (40, 41, 42). The signal for localization for many of the mRNAs which have been studied resides in the 3' untranslated region (32,42). Given that knowledge is limited as to how and why some mRNAs are localized to specific sub regions of the cytoplasm, for the majority of targets it is difficult to design ribozymes which will be at the right place in the cell to maximize interactions with a given target RNA. Kislauskis, et al. (42) have demonstrated that the mRNAs encoding two actin isoforms, β-cytoplasmic and α-cardiac, can occupy different cytoplasmic compartments within the same cytoplasm of chicken fibroblasts. Moreover, the sequences in the respective actin 3' UTRs were sufficient to localize a lac Z mRNA to the same cytoplasmic compartments. Actin isoforms contain very few differences in amino acid coding sequences, but the 3' UTR's are isoform specific, and evolutionarily conserved within a given isoform family, suggesting an important functional role (43). In order to demonstrate the utility of co-localizing a ribozyme transcript with a given mRNA, the β-actin and α-actin UTR's may be used to test their potential for co-localizing ribozyme and target mRNA's intracellularly. A similar approach

involves using the HIV-1 3′ UTR, which is present in all HIV transcripts.

The basic strategy is to incorporate the 3′ UTR of interest onto a reporter construct as well as to incorporate the same UTR onto a ribozyme transcript. The 293 and HeLa cell lines were used for the studies. The reporter construct to be used is depicted below and contains the human growth hormone (GH) gene driven by the SIV-1 LTR promoter. This system produces a readily quantifiable (using a radioimmunoassay) secreted protein. The linear range of response of GH expression to plasmid concentration in the 293 cell line was established. The expression of this construct is not dependent upon TAT expression, although a 10 fold stimulation of expression in the presence of SIV TAT was observed. If the results look promising in the 293 cell line, confirmation testing in HeLa cells will be carried out. The 3′ UTRs will be appended to both the growth hormone and ribozyme expression cassettes. To do this, the human β-actin or α-actin 3′ UTRs will be isolated from human genomic DNA or mRNAs utilizing PCR.

The primers for isolating the two human actin 3′ UTRs are:

beta actin oligo 5′
5′AGATCTTCTAGACCCGGGTAGGCGGACTATGACTTAGTTGC3′
(SEQ ID NO. 3)

beta actin oligo 3′
5′GAATTCGCTAGCTACGTACCCACCCTCTGCTGCCCCCAAC3′
(SEQ ID NO. 4)

alpha actin oligo 5′
5′AGATCTTCTAGACCCGGGCTAAGATGCCTTCTCTCTCCATC3′
(SEQ ID NO. 5)

alpha actin oligo 3′
5′GAATTCGTCAGCTACGTAACAATGCTCAGGGTGTCAAAGCA3′
(SEQ ID NO. 6)

The ribozyme will be expressed utilizing the RSV promoter with the appropriate actin UTR appended to the 3′ end. Utilizing transient transfection of the reporter constructs into pools of stably transfected ribozyme containing cells, the effect of the ribozyme mediated inhibition of the reporter construct was monitored. Ribozyme constructs may be made in the adeno associated virus vector backbone. The constructs will be encapsidated in collaboration with Saswati Chatterjee's laboratory, and transduced into three A293 or HeLa cell lines. Stable lines will be selected from G418, and levels of ribozyme expression will be monitored via primer extension and northern gel analyses. For each ribozyme, a non-cleaving mutant control will be used. The controls for 3′ UTR effects will utilize comparison of the efficiency of reporter gene inhibition as a function of having the β- versus α-actin 3′ UTRs, which localize to different intracellular compartments, appended to the reporter and ribozyme transcripts. Several ribozyme targets in the SIV leader region have been established which will be tested in conjunction with the UTRs. These ribozymes have been tested for substrate interaction using an in vitro gel shift assay, and identified by this process sites in the SIV LTR which are most accessible to binding. In each case where binding was shown to be efficient, good cleavage activity by the ribozyme was observed.

At this time, aside from the well known actions of Rev on RRE containing transcripts, there is very little known about the role, if any, of the HIV UTR on intracellular partitioning of messenger RNAs. The nucleotide sequence of the region is uninformative, but the functions of the LTR, such as transcription termination and polyadenylation signaling, must be conserved. Since the 5′ and 3′ LTRs of retroviruses are identical, but have functionally different roles

(transcription initiation for the 5′ LTR and termination and 3′ processing for the 3′ LTR), it is reasonable to ask whether placing a segment of the LTR at the 3′ end of a heterologous transcript will result in its functioning as a transcriptional termination, polyadenylation signal. An intact HIV-1 LTR has been appended to the 3′ end of an insulin reporter gene and more than 98% of the transcripts were correctly processed and polyadenylated at the authentic poly A site (44, 45). It is therefore reasonable to test this region for its potential use as an mRNA localization signal. The following experiments are illustrative.

The first set of sequences appended to the GH reporter construct included the last 20 bases of the pNL4-3 proviral<u>nef</u> coding sequence and extended to the 3′ terminus of the LTR. Much of this region is included in all of the viral messenger and full length genomic transcripts. This sequence contains the poly A additional signal and putative transcriptional termination region (45), but most importantly lacks cis-acting regulatory signals such as the RRE, INS and CRS. This region was isolated using PCR primers and appended to both the GH reporter gene construct and the ribozyme transcriptional units as described above.

The control constructs included the AAV poly A and termination signals, which were appended to the ribozyme and GH reporter constructs as well as mutant, non-cleaving ribozymes. Again, efficacy was measured by inhibition of growth hormone secretion in transient transfection assays of the GH construct into stable cell lines expressing the ribozyme constructs as described above.

c. Co-localization of anti-HIV-1 ribozymes with full length viral transcripts via the dimerization domain and the viral RRE.

The third strategy for co-localizing ribozyme and target RNAs will capitalize upon the unique RNA-RNA interaction of the dimerization domain of HIV (which is facilitated by the NCp7 nucleocapsid protein) (46-49) in combination with the RRE (to force cytoplasmic translocation of the ribozyme containing transcripts). The rationale for these studies is that ribozyme containing RNAs which harbor the signals required for packaging can be co-localized with unspliced viral mRNAs and genomic RNAs via interactions of the dimerization domains. The most probable targets for ribozyme interactions will be full-length viral RNAs, destined for encapsidation or translation into viral structural proteins. These experiments are based upon the success of a somewhat similar strategy employed by Sullenger and Cech (33). See Figure 8.

Genetic fusions consisting of the entire mature coding sequence or 18 bases of the 3′ end of human $tRNA^{LYS}$ were fused to hammerhead ribozyme containing RNAs with base pairing capabilities to the HIV-1 sequences immediately 5′ or upstream of the primer binding site. The 3′ terminal 18 nucleotides of the $tRNA^{LYS}$ are complementary to the primer binding site.

These chimeric molecules have been tested in cell free assays for their ability to bind to HIV-1 reverse transcriptase and their inhibitory activity on HIV-1 reverse transcriptase polymerization activity. The ribozyme moiety targets the cleavage of HIV-1 viral RNA at a known hammerhead cleavage site immediately upstream of the primer binding site for initiation of reverse transcription in the HIV-1 viral RNA. The site chosen for initial study, and reported here is an AUC in which cleavage is immediately after the C. This site is

absolutely conserved in all HIV-1 isolates sequenced to date. The chimeric RNAs, which are specifically bound by HIV-1 reverse transcriptase, should be carried into newly formed HIV-1 virions during viral assembly. The chimeric primers effectively block HIV-1 revere transcription, making them a novel, highly target specific, and unique anti-HIV-1 therapeutic agent. In addition, the $tRNA^{LYS}$ portion contains within its mature coding sequence the elements required for transcription by human RNA polymerase III, thereby making it feasible to insert the gene, rather than the RNA, into human cells.

Studies of the binding of the chimeric molecules to HIV-1 reverse transcriptase revealed that the complex of chimeric $tRNA^{LYS}$-ribozyme, or 18 3′ nucleotides of $tRNA^{LYS}$-ribozyme, or $tRNA^{LYS}$ with an extra 6 nucleotides appended to the 3′ end, when base paired to the primer binding site signal of HIV-1 RNA, serves as a substrate for a novel ribonuclease activity associated with HIV-1 reverse transcriptase. This activity results in cleavage of the primer at a site very close to the 3′ end of the $tRNA^{LYS}$ molecule, CCA-3′. This activity is of unknown function in the viral replication cycle, but may play an important role in the use of chimeric RNAs by freeing the ribozyme moiety from the tRNA moiety such that it can cleave one or both of the viral RNAs encapsidated in the HIV-1 virion.

GENERAL PURPOSE OR UTILITY OF THE INVENTION

Co-equalization mechanisms and the resulting living cells which include co-equalized inhibitors and targets are disclosed. HIV and other lentiviral RNAs co-equalized with a ribozyme provide intracellular and therapeutic agents and vaccines for mammalian lentiviral infections. Such therapeutic agents and vaccines are administered in known manner by viral mediated delivery, e.g., AAV or retroviral deliveries.

The idea of chimeric $tRNA^{LYS}$-ribozyme molecules which effectively compete with $tRNA^{LYS}$ for binding to HIV-1 reverse transcriptase is novel. It provides a possible mechanism for specifically delivering inhibitors of HIV-1 reverse transcriptase to the virion particle itself. Such inhibitory agents will render these viral particles non-functional, and thus serve as highly specific, non-toxic therapeutic agents.

It has been demonstrated that the entire $tRNA^{LYS}$ molecule, as well as various segments of the tRNA itself, are capable of specifically interacting with HIV-1 verse transcriptase. No one has shown that chimeric molecules such as the ones described could specifically bind to HIV-1 reverse transcriptase polymerase activity. There is one published report of an RNAse cleavage activity associated with HIV-1 reverse transcriptase. This activity was only shown to cleave HIV-1 RNA, not the primer. This activity cleaves twice in the primer binding site, and only substrates paired with $tRNA^{LYS}$.

The RNA attached at the 3′ end of the $tRNA^{LYS}$ need not be a ribozyme, but any extra RNA which can base air with the HIV-1 target upstream of the primer binding site. If a ribozyme is joined to the tRNA, other cleavage sites such as CUC, or CUA which are on the HIV-1 sequence just to the 3′ side (downstream) of the AUC site, can be targeted. It is not necessary to make an entire $tRNA^{LYS}$-ribozyme fusion because it is now known that the last 18 nucleotides of $tRNA^{LYS}$ fused to the ribozyme are also bound by HIV-1 reverse transcriptase. Genetic variants of $tRNA^{LYS}$ which compete better than $tRNA^{LYS}$ for binding to HIV-1 transcriptase are included in the invention.

The ribozyme fusions to $tRNA^{LYS}$ allow specific targeting of the ribozyme to HIV-1 virion. Since all retroviruses use cellular tRNAs for priming, this invention provides a general strategy for inhibiting

other retroviruses as well. Existing ribozyme technology makes use of specific base pairing between ribozyme and target, but this is accomplished by diffusion of the ribozyme until it finds a target RNA. This invention uses well known retroviral packaging pathways to specifically carry the ribozyme into the virion, and get it bound to the correct site on the viral RNA for cleavage.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: John J. Rossi
Garry P. Larson

(ii) TITLE OF INVENTION: Inhibitors and Target Molecule Co-Localization

(iii) NUMBER OF SEQUENCES: 6

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(F) ZIP: 91010-0269

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Wang Double Density 5 1/4" diskette

(B) COMPUTER: Wang PC

(C) OPERATING SYSTEM: MS-DOS (R) Version 3.30

(D) SOFTWARE: Microsoft (R)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 02 December 1994

(C) CLASSIFICATION: Unknown

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(A) APPLICATION NUMBER: 08/185,827

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Irons, Edward S.

(B) REGISTRATION NUMBER: 16,541

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(B) TELEFAX: (202) 785-5351

(C) TELEX: None

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

UGGAAAAUCU CUAGCAGUGG CGCCCGAACA GGGAC 35

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 130

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

GGCGCCUUUU UACCGUUUAA AGCAGGAGUG CCUGAGUAGU CAGAUCGUCA 50
CCGCGGGCUU GUCCCUGAAC UUGGGACCUG GGAGUCUAAU UUUCAGACUA 100
CGAGAUGGCU GACUCGAUAG GCCCGAGCUC 130

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

AGATCTTCTA GACCCGGGTA GGCGGACTAT GACTTAGTTG C 31

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

GAATTCGCTA GCTACGTACC CACCCTCTGC TGCCCCCAAC 30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:

AGATCTTCTA GACCCGGGCT AAGATGCCTT CTCTCTCCAT C 31

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleotides

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6:

GAATTCGTCA GCTACGTAAC AATGCTCAGG GTGTCAAAGC A 31

CLAIMS:

1. A process which comprises the positioning, within a living cell, of a target molecule and an inhibitor for said target molecule, said positioning being such that the concentration of the inhibitor molecule with respect to the target molecule is enhanced.

2. The claim 1 process in which the target molecule is an RNA molecule and the inhibitor is a ribozyme.

3. The claim 1 process in which the target molecule is an HIV-1 RNA molecule and the inhibitor is a ribozyme which cleaves said HIV-1 RNA molecule.

4. The method which comprises co-localizing a target molecule and an inhibitor for said target molecule within a living cell.

5. A living cell in which a target molecule and an inhibitor for said target molecule are co-localized.

6. The claim 4 method in which the target molecule is an RNA molecule and the inhibitor is a ribozyme which cleaves said RNA molecule.

7. The living cell of claim 5 in which the target molecule is an RNA molecule and the inhibitor is a ribozyme which cleaves said RNA molecule.

8. A method which comprises co-localizing within a living mammalian cell

an RNA target molecule, and

a ribozyme which cleaves said RNA target molecule

said ribozyme including

(i) the dimerization or packaging signal of said RNA target molecule, or

(ii) a sequence capable of pairing with said RNA molecule at a site upstream of the primer binding site to the 3′ end of $tRNA^{LYS3}$, or

(iii) a 3′ untranslated region (UTR) of said RNA molecule.

9. The claim 8 method in which said RNA target molecule is an HIV-I RNA molecule.

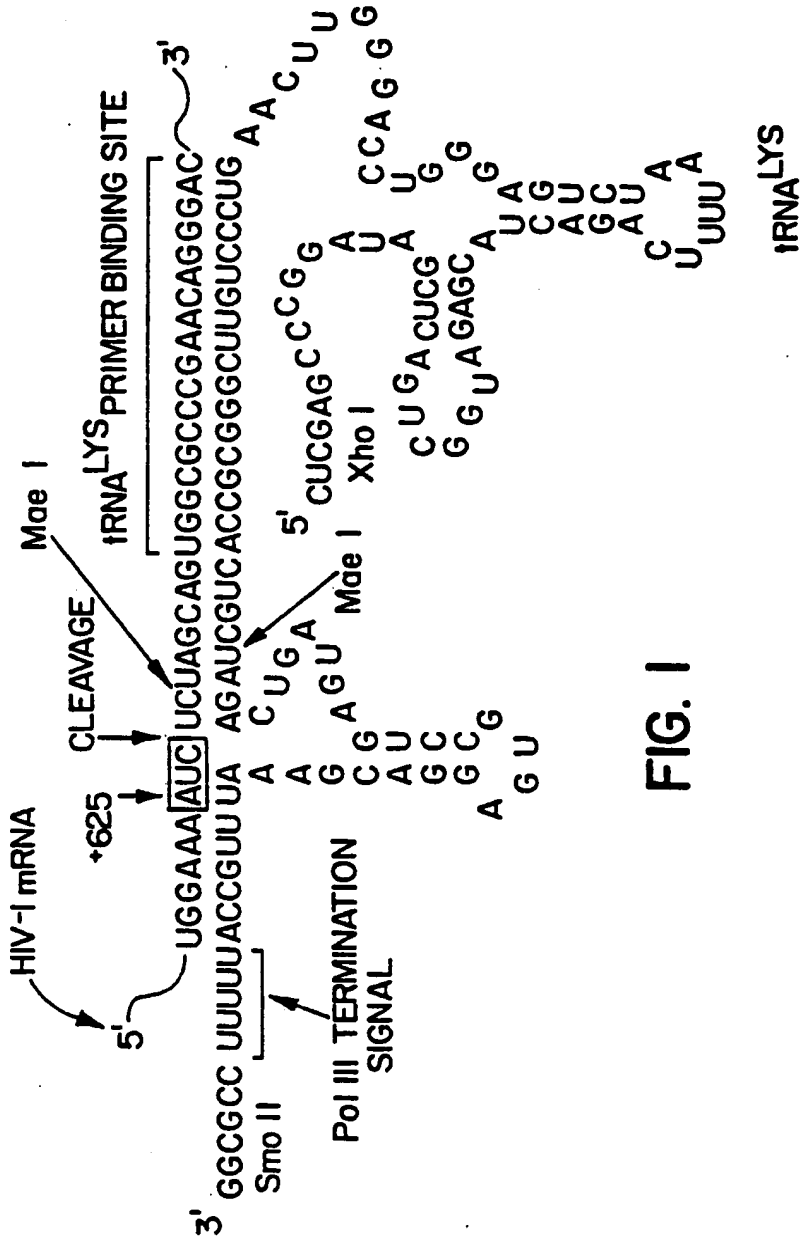
HIV-1 mRNA
5'
UGGAAAAUCUUUUACCGUUUA
3' GGCGCC
Smo II
+625
CLEAVAGE
Mae I
tRNA^LYS PRIMER BINDING SITE
AUC
UCUAGCAGUGGCGCCCGAACAGGGAC
AGAUCGGUCACCGCGGGCUUGUCCCUG
3'
Pol III TERMINATION SIGNAL
Mae I
Xho I
5' CUCGAG
tRNA^LYS

FIG. 1

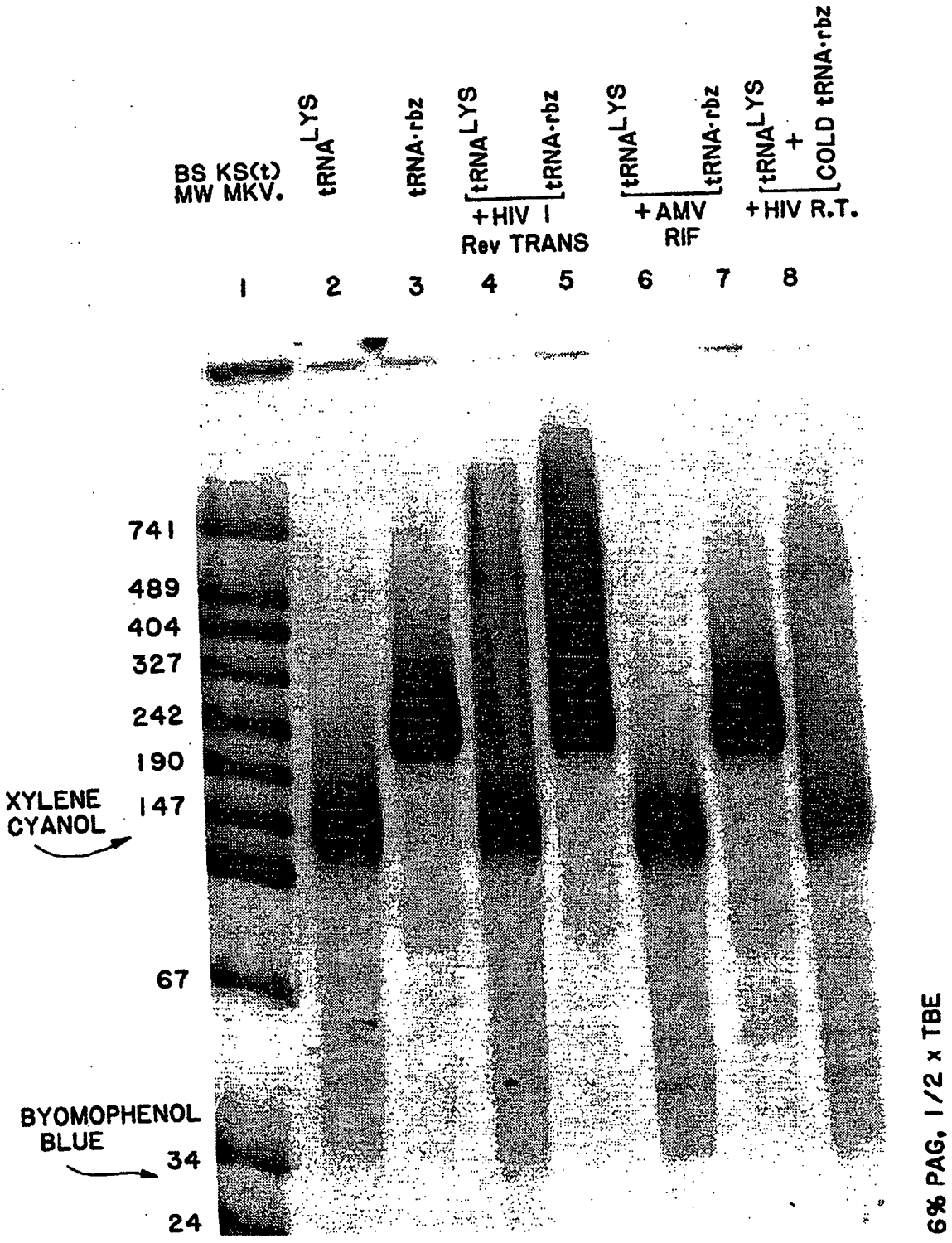
BS KS(t) MW MKV.
tRNA LYS
tRNA·rbz
tRNA LYS + HIV I Rev TRANS
tRNA·rbz
tRNA LYS + AMV RIF
tRNA·rbz
tRNA LYS + COLD tRNA·rbz + HIV R.T.
1 2 3 4 5 6 7 8
741
489
404
327
242
190
147
67
34
24
XYLENE CYANOL
BYOMOPHENOL BLUE
6% PAG, 1/2 x TBE

FIG. 2

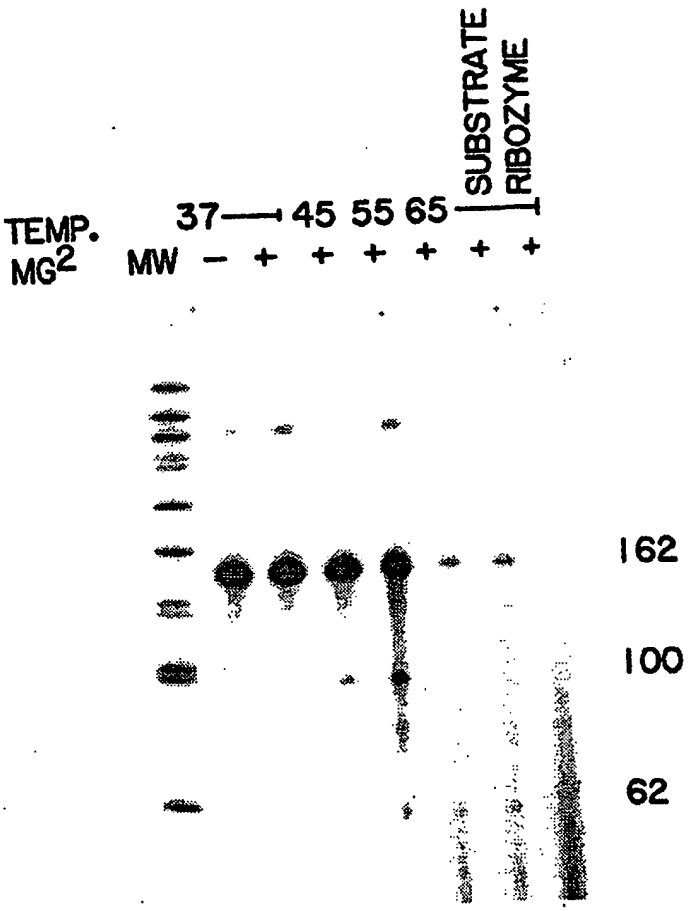

FIG. 3

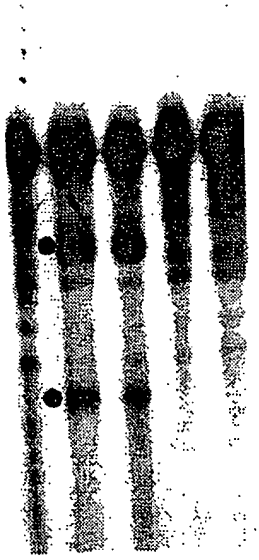

FIG. 4

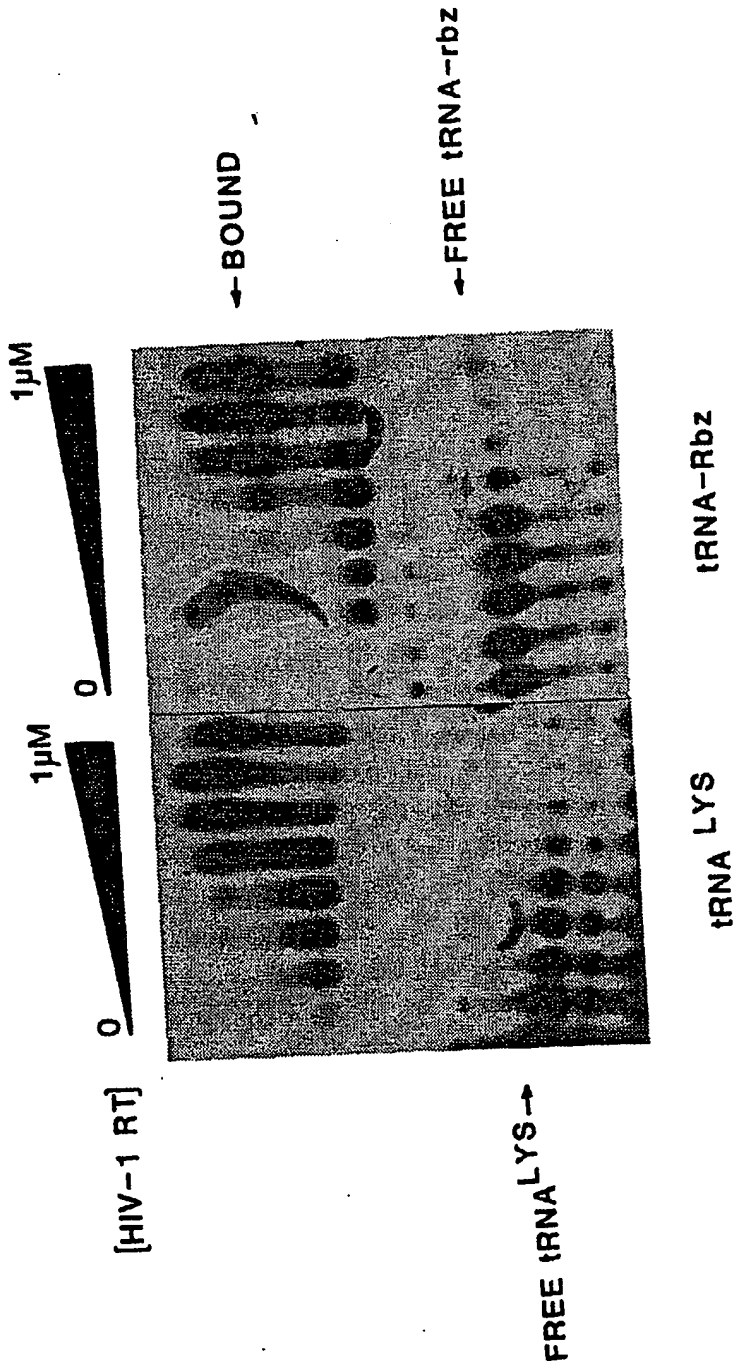
←BOUND
←FREE tRNA-rbz
1μM
0
1μM
0
[HIV-1 RT]
FREE tRNA LYS→
tRNA LYS
tRNA-Rbz

FIG. 5A

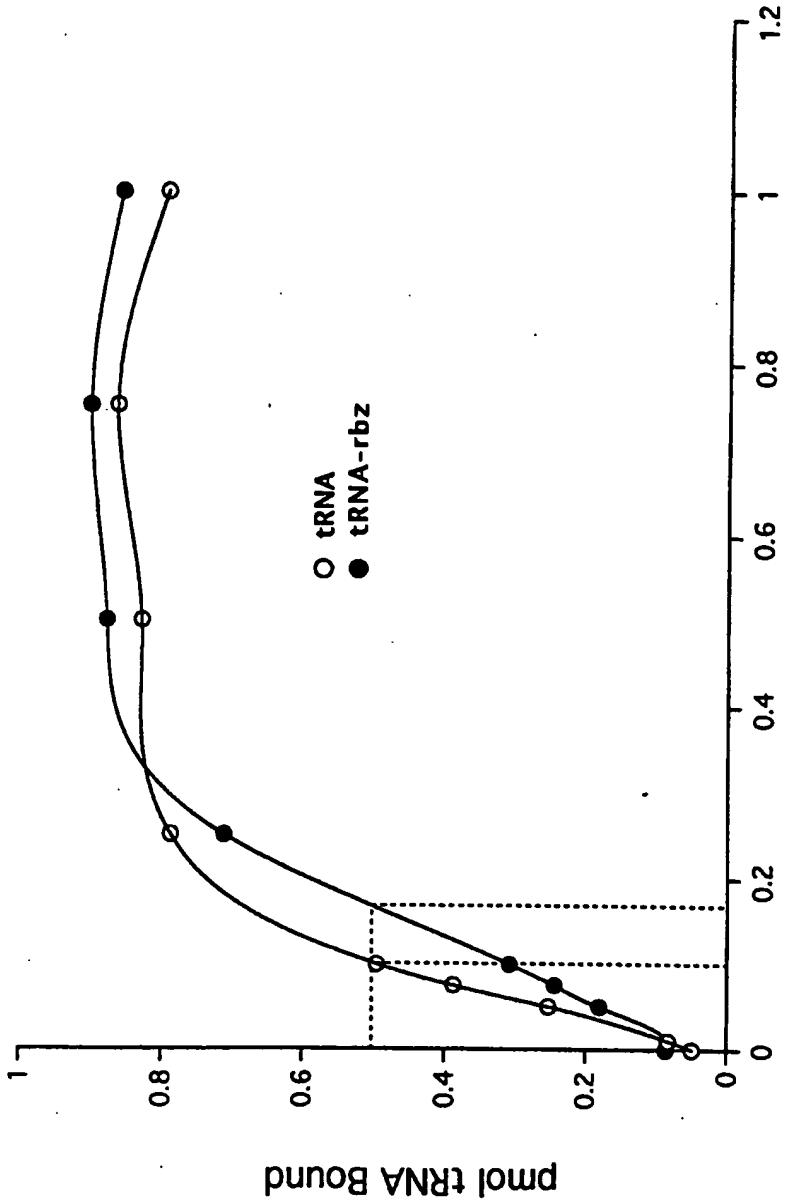
pmol tRNA Bound
0
0.2
0.4
0.6
0.8
1
0
0.2
0.4
0.6
0.8
1
1.2
[RT] (μM)
tRNA
tRNA-rbz

FIG. 5A-1

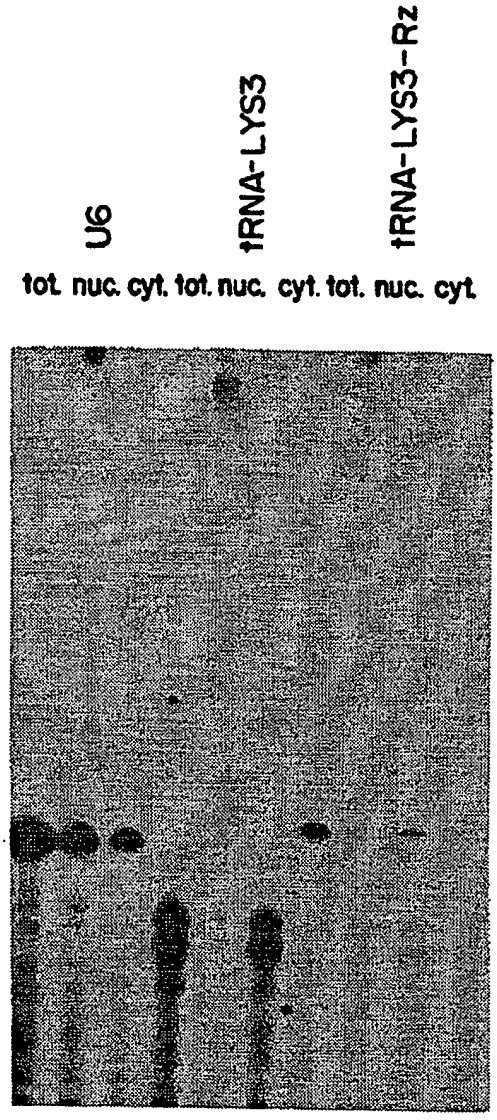

FIG. 5B

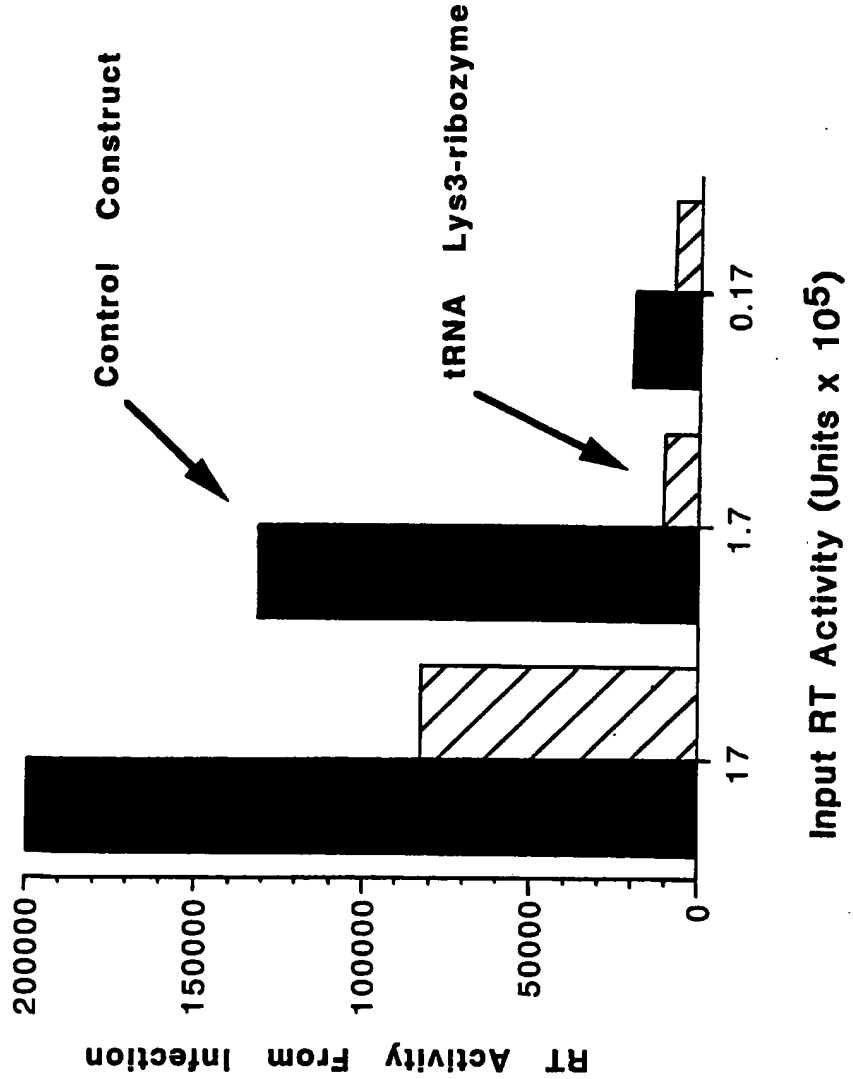
Control Construct
tRNA Lys3-ribozyme
RT Activity From Infection
200000
150000
100000
50000
0
17
1.7
0.17
Input RT Activity (Units x 10^5)

FIG. 5C-1

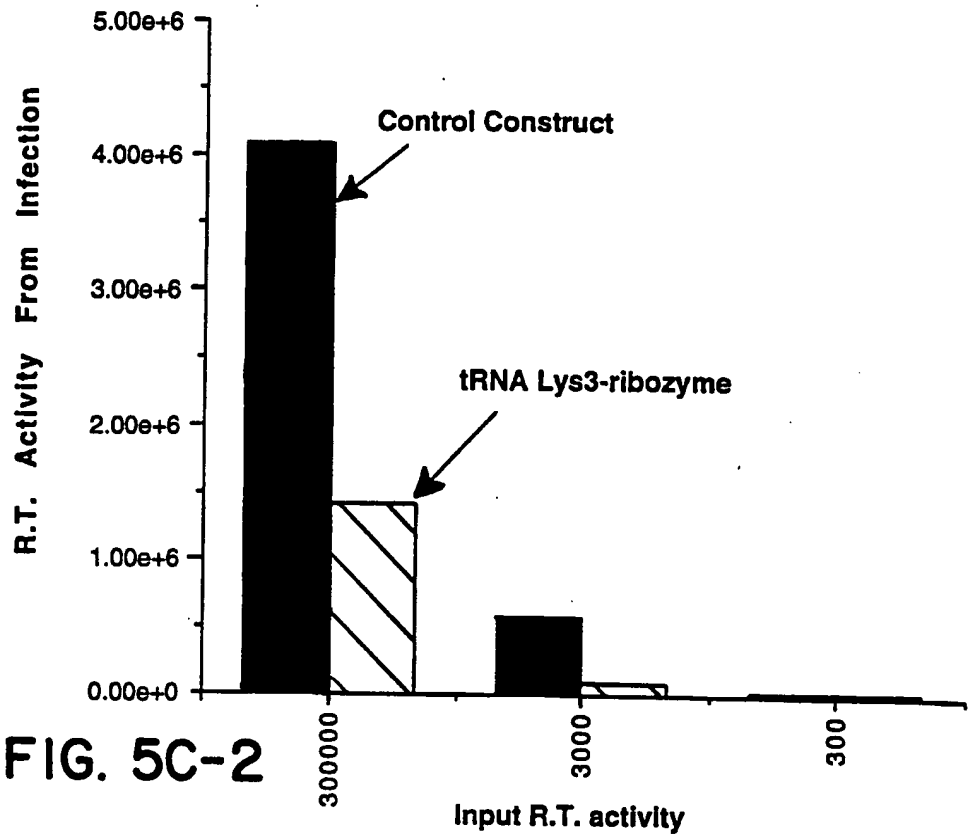

FIG. 5C-2

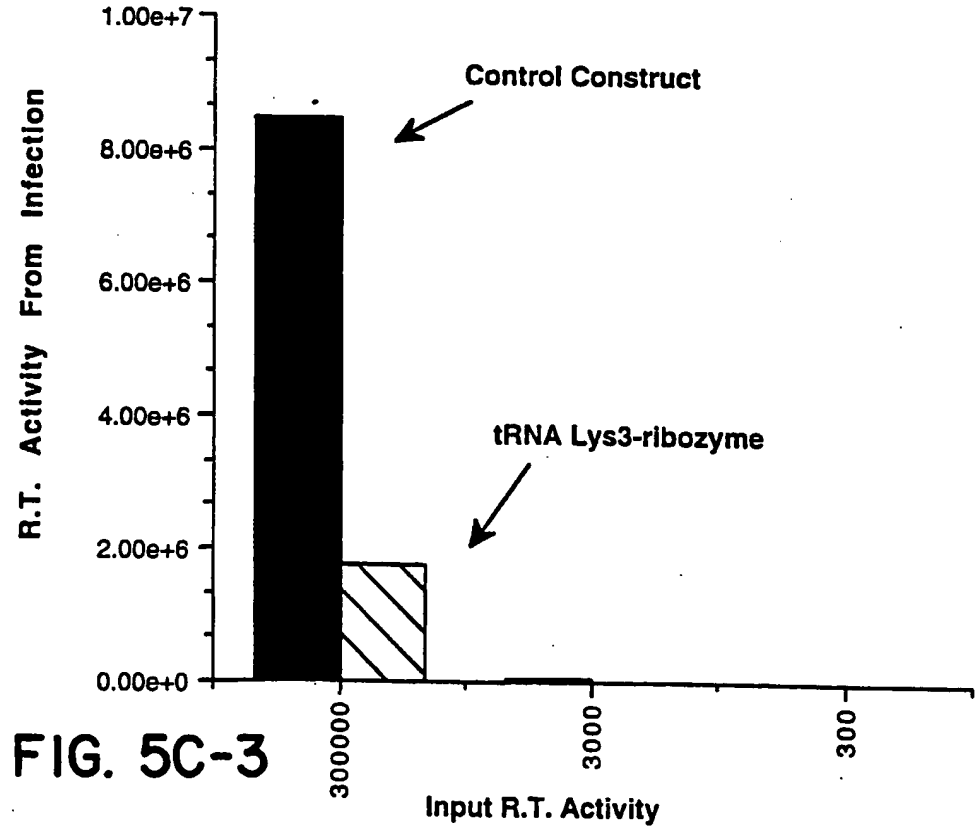

FIG. 5C-3

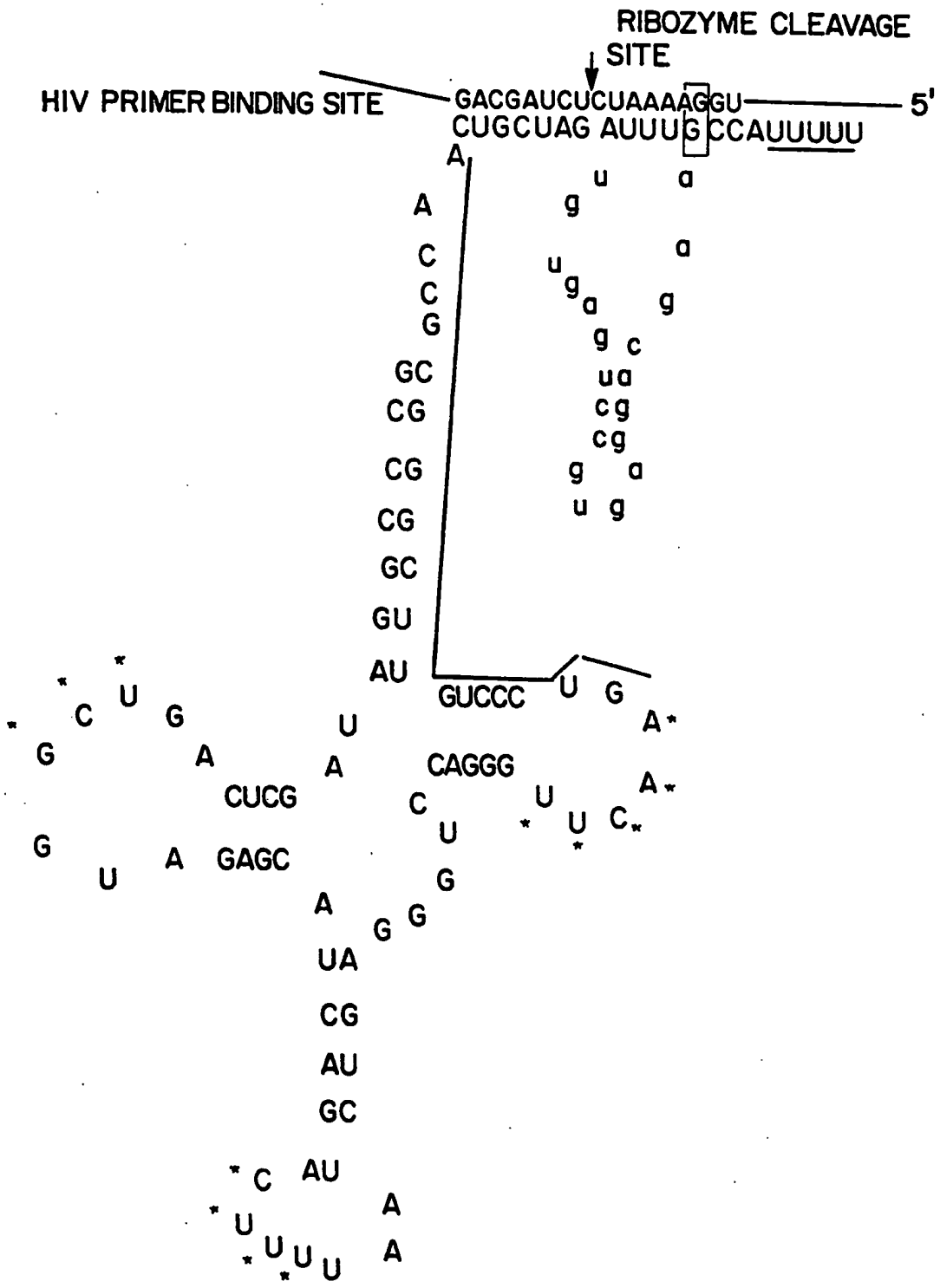
RIBOZYME CLEAVAGE SITE
HIV PRIMER BINDING SITE
GACGAUCUCUAAAAGGU
5'
CUGCUAG AUUUGCCAUUUUU
GUCCC
CAGGG
CUCG
GAGC

FIG. 6

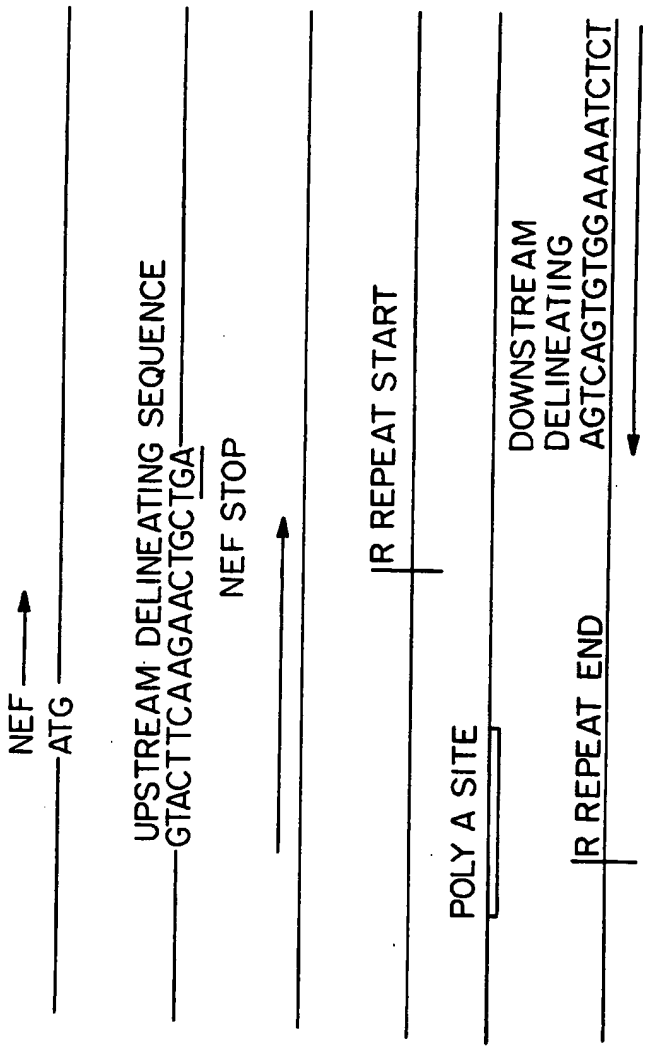
NEF
ATG
UPSTREAM DELINEATING SEQUENCE
GTACTTCAAGAACTGCTGA
NEF STOP
IR REPEAT START
POLY A SITE
DOWNSTREAM
DELINEATING
AGTCAGTGTGGAAAATCTCT
IR REPEAT END

FIG. 7

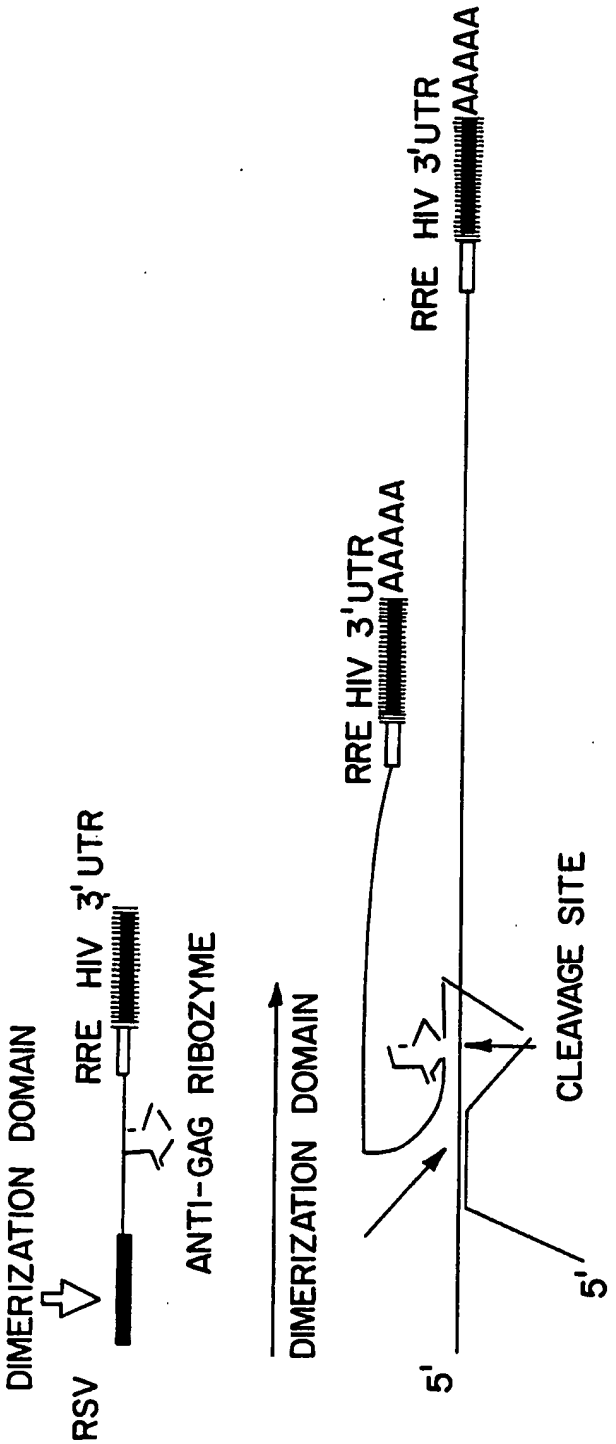
DIMERIZATION DOMAIN
RSV
RRE HIV 3'UTR
ANTI-GAG RIBOZYME
DIMERIZATION DOMAIN
RRE HIV 3'UTR
AAAAA
RRE HIV 3'UTR
AAAAA
CLEAVAGE SITE
5'
5'

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13798

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/43, 47/48; C12N 9/22, 5/22; C12Q 1/25
US CL :435/6,91.31,240.2; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6,91.31,240.2; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog: BIOSIS, MEDLINE, Derwent Biotechnology Abstracts, CAS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Journal of Biological Chemistry, Volume 269, Number 20, issued 20 May 1994, Arts et al., "Comparision of deoxynucleotide and tRNA Lys-3 as primers in an endogenous human immunodeficiency virus-1 in vitro reverse transcription/template-switching reaction", pages 14672-14680, see entire document.	1-9
X -- Y	Biochemical and Biophysical Research Communications, Volume 192, Number 2, issued 30 April 1993, Wisotzkey et al., "Cleavage of cottontail rabbit papillomavirus E7 RNA with an anti-E7 ribozyme", pages 833-839, see entire document.	1 ----- 2-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 FEBRUARY 1995	Date of mailing of the international search report 09 MAR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer John L. LeGUYADER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int..ational application No. PCT/US94/13798

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 247, issued 09 March 1990, Sarver et al., "Ribozymes as potential anti-HIV-1 therapeutic agents", pages 1222-1225, see entire document.	2-7